Targeting therapeutic T cells to the bone marrow niche

A thesis submitted to University College London for the degree of doctor of philosophy

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Declaration:

I, Anjum Bashir Khan, confirm that this thesis is my own work, except where specifically stated, and has not been submitted in whole or in part to any other university.

Abstract

Anti-cancer immunotherapies aim to mediate a specific response targeting malignant cells without accompanying damage to normal tissue associated with conventional therapies, but induction of T cell differentiation and exhaustion enables successful tumour progression. In this thesis I will explore different means of enhancing the accumulation and function of therapeutic CD8 T cells, as a means of achieving functional cure through persisting immunological memory. I will show that the key features of T cell memory can be imprinted upon $CD8^+$ T cells by enhancing homing to specific organs, enabling privileged access to cell-mediated factors.

The interaction between the chemokine receptor CXCR4 and the ligand CXCL12/SDF-1 is required for successful homing of haematopoietic stem cells (HSCs) to stromal niches within the bone marrow (BM). The bone marrow is known to be a unique organ for immunological memory, including memory T cells. I hypothesised that replicating this bone marrow homing interaction in $CD8^+$ T cells would preferentially generate memory T cells. I demonstrate through in vivo imaging and flow cytometric analyses that T cells over-expressing CXCR4 accumulate preferentially in the BM near vascular-associated $CXCL12^+$ cells, retain a less differentiated central memory phenotype despite repeated antigenic stimulation, and produce enhanced effector cytokines on restimulation. Compared to control T cells, these cells

demonstrate lower expression of exhaustion and senescence markers, suggesting the capacity for long-term persistence after activation.

I go on to show that numerical accumulation and many of these functional attributes are dependent upon cell-extrinsic expression of IL-15Rα. T^{CXCR4} demonstrate heightened graft-versus-tumour effects in allogeneic bone marrow transplant models of B-cell lymphoma in comparison to control T cells. I provide evidence that this anti-tumour effect is mediated by enhanced functional capacity rather than numerical accumulation or out-competing immunosuppressive populations. In summary, this strategy offers a tractable means of enhancing T cell engraftment, persistence and function, with potential for cross-platform therapeutic applications including anti-cancer immunotherapy.

Impact Statement

Immunotherapy is currently upending conventional ideas regarding optimal treatment of malignancies, which have largely exhausted the possibilities of chemotherapy and radiotherapy. A large number of trials have been initiated to explore the potential of various agents, yet thus far results have not fulfilled high expectations.

The two most promising and advanced approaches currently involve the use of genetically modified chimeric-antigen receptor T cells (CAR-T cells) and checkpoint inhibiting antibodies (CPI). These approaches display dramatic responses in subsets of patients, but are yet to be widely applicable to the majority of patients who still receive conventional therapy. For these approaches to become more widely applicable, three key issues need to be tackled. Firstly, current approaches to generate sufficient numbers of modified T cells are expensive, require substantial preparation time and fail to generate sufficient cells in a substantial proportion of patients. Secondly, tumours generate intrinsic mechanisms of resistance to the immune system, preventing T cells from homing to the tumour niche or prematurely exhausting them to limit potency. Thirdly, current adoptive cell therapy often uses terminally differentiated T cells with limited potential for long-term immunosurveillance: the median time to relapse for adults with acute leukaemia receiving CAR-T cells is just six months (1).

The approach detailed in this thesis aims to combat these three problems through a unified strategy. The formation of immunological memory is known

to be critical for long-term immune surveillance including our most effective vaccines (2). The bone marrow is important for the generation of long-lived memory T cells with enhanced protection against tumours (3, 4). These highly potent central memory T cells are able to reconstitute the entire T cell immune response while renewing themselves akin to stem cells in other organs. The magnitude of these cells within a CAR-T cell product correlates with the anti-tumour response. The ability to reliably generate early memory T cells is the focus of intensive research efforts, but proven methods to maintain their phenotype in vivo are lacking (5, 6).

In this project we demonstrate the ability to generate large numbers of these early memory cells and maintain them over extended periods in living animals despite repeated exposure to antigen. We do this by means of enhanced homing to the bone marrow niche, which enables privileged exposure to a potent memory-generating cytokine, IL-15. In other words, these T cells are able to access a molecular pharmacy to create large numbers of powerful immune cells. These cells are capable of generating long-lasting anti-tumour immunity addressing all three of the challenges described above: namely engraftment, persistence and self-renewal. This strategy is applicable not just to CAR-T cells, but to the range of adoptive T cell therapies, including the potentially game-changing ability of T cell receptor-gene modified cells to target mutations in any tumour. This tractable method could also potentially be harnessed for treatment of a variety of autoimmune diseases such as diabetes and rheumatoid arthritis, which cause enormous morbidity, mortality and financial burdens, through regulatory T cell expansion. This work has been disseminated through presentations at national and international

conferences, and publication in a high-impact peer reviewed open-access journal, and is the subject of a patent application for translational work.

Table of Contents

Table of tables

Table of Figures

Acknowledgements

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1 Introduction

1.1 Chemokines and chemokine receptors

The chemokines are a group of small, secreted proteins united by their ability to induce cell migration. They are distinguished from other cytokines by their ability to bind to G-protein coupled chemokine receptors (GPCRs) but can be more readily conceptualised into inflammatory (inducible) and homeostatic (developmentally regulated) subgroups, although this useful division can be fluid and several receptors fit into both categories (Table 1). Members of the homeostatic group are produced constitutively and have numerous roles in lymphoid organogenesis, limb development, stem cell maintenance, angiogenesis and even metastasis (7, 8).

Despite considerable sequence variability, almost all chemokines share a characteristic tetracysteine motif with two disulphide bridges. This motif forms a fold comprising three β strands, a carboxy (C) terminal α-helix and an amino (N) terminal region. The four chemokine subtypes are named according to the location of the first two-cysteine groups: the CXC-chemokines (α subclass), the CC-chemokines (β subclass), C-chemokines and CX_3C-chemokines, where X denotes an amino acid (9).

Chemokine receptors also share a characteristic structure. They are almost all composed of seven-transmembrane polypeptide chains containing three extracellular and three intracellular loops, an extracellular N terminal domain

Chemokine	Chemokine Partners	Group	Function	Disease Association
CXCR1	CXCL8 (IL-8), CXCL6 (GCP-2)	I	Neutrophil trafficking	Sepsis Atherosclerosis
CXCR2	CXCL7, CXCL5, CXCL1, CXCL2, CXCL3, CXCL8	I	Neutrophil trafficking	Sepsis Atherosclerosis
CXCR3	CXCL9, CXCL10, CXCL11	I	T_H1 response, NK and $CD8^+$ T cell trafficking	MS, RA, transplant rejection, GVHD
CXCR4	CXCL12 (SDF-1)	Dual	Bone marrow homing, cardiac, immune and neurological development	Cancer metastasis, angiogenesis, HIV, IBD, RA, nephritis
CXCR5	CXCL13	H	B cell and T_{FH} localisation in mature lymph nodes	
CXCR6	CXCL16	H	NK, NKT, ILC and T^{RM} migration	
CCR1	CCL5 (RANTES) CCL3 (MIP-1α), CCL7 (MCP-3)	I	Macrophage and NK, T cell migration, T-DC interaction	MS, transplant rejection, RA, asthma, nephritis
CCR2	CCL2 (MCP-1), CCL8 (MCP-2), CCL7, CCL13 (MCP-4)	I	Monocyte and T cell trafficking	MS, RA, transplant rejection, asthma, atherosclerosis
CCR3	CCL11, CCL28, CCL5	I	Eosinophil and basophil migration	Asthma
CCR4	CCL17, CCL22	I	T_H2 response and migration, T_{reg} migration	Asthma
CCR5	CCL4 (MIP-1β), CCL5, CCL3	I	Macrophage and NK migration, T-DC interaction	MS, RA, asthma, nephritis, IBD, HIV, transplant rejection, GVHD
CCR6	CCL20 (MIP-3α)	Dual	T_H17 responses, B and DC gut	

			homing	
CCR7	CCL19, CCL21	Dual	T and DC LN homing	
CCR8	CCL1	Dual	T_H2 and T_{reg} trafficking	Possibly asthma
CCR9	CCL25	H	T cell gut homing, thymocyte migration	
CCR10	CCL28, CCL27	H	T cell and IgA plasma cell mucosal/skin homing	
CCR11	CCL25	I	T cell gut homing, thymocyte migration	
CX_3CR1	CX_3CL1 (Fractalkine), CCL26	D	NK, monocyte and T migration	Immune evasion
XCR1	XCL1 (Lymphotactin)	D	$CD8^+$ DC cross-presentation	

Table 1 Chemokine receptors and their ligands
Major homeostatic and inflammatory chemokine receptors and their partners, grouped via subclass: α (CXC, blue), β (CC, red), CX_3C (green) and C (black). I denotes Inducible, H Homeostatic, D Dual, T_H1 T-helper 1 cell, NK natural killer cell, NKT natural killer T cell, ILC innate lymphoid cell, T_H2 T-helper 2 cell, T_H17 T-helper 17 cell, T_{reg} regulatory T cell, T^{RM} tissue-resident memory T cell, DC dendritic cell, MS multiple sclerosis, RA rheumatoid arthritis, IBD inflammatory bowel disease.

and a serine/threonine-rich intracellular C-terminal domain. The external structure allows for specificity of ligand recognition while conserved transmembrane sequences, cytoplasmic loops and the C-terminal are relevant to signaling and receptor internalization. Chemokine monomers are thought to bind to GPCRs in a two-site model of receptor activation. The negatively charged receptor N-terminus and extracellular loops interact with the chemokine core domain (chemokine recognition site 1, CRS1) while the

chemokine N-terminus (triggering domain) interacts with the ligand-binding pocket of the receptor (chemokine receptor site 2, CRS2) (10), resulting in receptor activation. This model is supported by evidence that truncation mutations in chemokine N-termini lead to loss of signaling activity despite ongoing receptor binding (11). Further evidence for this paradigm is demonstrated by the ability of the small molecule antagonist AMD3100 to dislodge the CXCL12 N-terminus from its binding pocket in the CXCR4 transmembrane region (CSR2) without displacing the binding of the chemokine core domain to the CXCR4 extracellular region (12); this activity has been exploited for mobilization of haematopoietic stem cells (see below).

The chemokine-receptor system displays substantial flexibility as evidenced by the existence of several ‘silent’ non-signaling receptors historically thought to regulate immune reactions by acting as decoy or scavenger receptors to restrict migration. These molecules do not initiate classical chemokine receptor signaling responses to ligand and are now referred to as atypical chemokine receptors (ACKR) in the new nomenclature, eg. Duffy antigen receptor for chemokines (DARC) is now known as ACKR1 and CXCR7 as ACKR3 (13). Upon binding to ACKRs, chemokines can be internalized and destroyed in lysosomes to deplete extracellular chemokines, transported intact across polarized cells, or taken up and released. For example, ACKR1 may act as a chemokine sink or buffer in times of abundance. The beta-arrestins are polyfunctional cytosolic adapter proteins initially known for desensitisation of GPCRs (14). CXCL12 binding to ACKR3 induces beta-arrestin relocalisation and hence signal transduction of CXCR4, modulating chemokine responses without activating G-protein signalling (15). In addition,

ACKRs can homodimerize or heterodimerize with conventional chemokine receptors, such as CXCR4, to alter signaling outputs (16, 17).

Soluble chemokines can interact with carbohydrate structures called glycosaminoglycans (GAGs) to promote cellular directionality. GAGs are attached to cell proteoglycans or shed into the extracellular matrix, and prevent chemokines being washed away by the bloodstream, enabling formation of chemokine gradients and facilitating local rather than paracrine action.

Many chemokines bind to multiple receptors and vice versa, potentially leading to high levels of redundancy. This redundancy is thought to provide evolutionary robustness in the face of pathogen evasion strategies and natural genetic polymorphisms within populations (18). However, this interpretation has been challenged by evidence of differential effects based upon cellular localisation within the body, and formation of chemokine receptor heteromers that allow for fine tuning of receptor signaling (9). Many chemokine receptors form oligomers during protein biosynthesis to facilitate protein folding (19). In contrast, only chemokine monomers are required for activation of receptors and oligomers must dissociate to bind GPCRs (20). Chemokines can adopt a wide variety of oligomerization structures to bind to GAGs, from dimers to polymers. Oligomerization can dramatically alter binding affinity; for example the binding affinity of CXCL4 and CCL5 monomers to chondroitin sulfate is lost in dimerization, while XCL1 interconverts between the canonical structure binding to XCR1 receptors and the GAG-binding dimer (21). Furthermore, activation of the same receptor by different ligands has differential effects, e.g. CCL19 and CCL21 both promote

calcium signaling via CCR7, but only CCL19 induces efficient phosphorylation and receptor internalization. This functional selectivity allows for modulation of important roles in cellular signaling and movement (9).

1.2 Physiological roles of chemokines

Chemokines are critical for the movement of all immune cells. This movement encompasses a range of roles from developmental migration and homeostasis to primary and recall responses of both the cellular and humoral branches of the immune system. In addition to the cardinal feature of chemotaxis, chemokines and their receptors can be subdivided by their ability to orchestrate cell migration under conditions of inflammation or establish homeostatic microenvironments conducive to immune cell growth and maintenance.

Pro-inflammatory chemokines are produced by a range of cells to recruit inflammatory cells to sites of injury, for example interleukin-8 (IL-8) produced by macrophages and endothelial cells primarily recruits neutrophils via CXCR1 and CXCR2, to induce phagocytosis and activation (22). The development of immune progenitor cells is exquisitely dependent upon expression of chemokine receptors. In the bone marrow, retention of haematopoietic progenitors and the development of the majority of their immune descendants including B cells, monocytes, macrophages and neutrophils is almost entirely dependent upon CXCL12/ CXCR4 interactions (23). As these cells develop, they downregulate CXCR4 to allow migration out

of the BM, in conjunction with upregulation of other signals such as CCR2 in monocytes and sphingosine-1-phosphate in B cells, to counteract the CXCR4 retention signal.

The vertebrate immune response requires that naïve T cells undergo thymic selection to eliminate or induce anergy of autoreactive cells. Following thymic selection, naïve T cells undergo activation and differentiation in response to cognate antigen in controlled molecular contexts. To enable successful recognition of the vast array of potential antigens, professional antigen-presenting cells and lymphocytes are preferentially guided to specific microenvironments within secondary lymphoid organs such as lymph nodes by chemokines (24). Chemokines therefore play a central role in the migration and hence development of lymphocytes, from progenitor stage to antigen exposure and differentiation, as detailed below.

Following haematopoietic development, T cell progenitors migrate from the BM to the thymus in response to CCL21, CCL25 and CXCL12 secreted by thymic stroma, and subsequently progress through intra-thymic development via expression of CCR7, CXCR4 and CCR9. Upon release into the circulation, they enter the high endothelial venules (HEVs), where in the absence of inflammation, stromal cells secrete CCL21 onto the luminal surface of the high endothelial venules (HEVs). Initial binding of lymphocytes to the endothelium is mediated by selectins, particularly L-selectin, expressed on most leucocytes. This initial tethering stage is converted to rolling adhesions due to the high shear forces involved. During this process naïve T cells expressing CCR7 engage with CCL19 and CCL21, triggering integrin activation and firm arrest (24, 25). A minor role is also played by CXCL12 produced by fibroblastic

reticular cells (FRCs), which interacts with CXCR4 on naïve T cells to further activate LFA-1 (lymphocyte function-associated antigen-1) and increase LN entry, a process upregulated in the context of an inflammatory stimulus (23, 26). Following arrest, T cells migrate through the endothelial and stromal layers, presumably following chemokine gradients, and migrate along paths parallel to the course of the FRC network, allowing optimal scanning of multiple dendritic cells (DCs). Lymphatic endothelium secretes CCL21 to guide DC migration and in turn, DCs secrete CCL3, CCL4, CCL5 and CCL17 to attract naïve T cells and promote T cell priming (23).

When considering cell migration, a distinction is drawn between migration along a gradient of immobilised chemoattractant (haptotaxis), migration driven by soluble chemokines without a directional gradient (chemokinesis) and migration driven by a gradient of soluble chemokines (chemotaxis) (27). Basal motility for naïve T cells within lymph nodes is maintained by chemokines such as CCL19 and CCL21, fitting the pattern of chemokinesis. In the inflammatory setting, activated and memory T cells express a range of chemokine receptors and ligands such as CXCR3 and XCL1 in response to microenvironmental cues, shaping multicellular encounters in a model more consistent with the concept of chemotaxis and amplifying the memory response (27).

T cell movement was initially thought to resemble a random walk, ie. stochastic movements of seemingly chaotic steps interspersed with pauses (28). This concept is known as a Brownian walk, analogous to the random motion of particles suspended in a fluid colliding with other molecules. Inflammatory T cell motion within the brain has also been described as

resembling a Lévy walk, named for the French mathematician who described a pattern of many small moves associated with a few longer movements. This pattern, widely found in the biological world from hunter-gatherers to honeybees (29, 30), is more efficient when seeking rare targets. In the same setting CXCL10 enhanced speed in response to pathogens, shortening the time to antigen recognition (31). This finding has not been replicated within the steady-state lymph node, where heterogeneous migration modes have been observed, and debate continues over the role of the microenvironment, possible interference of long Levy walk movements with optimal DC scanning, and variation in statistical approaches used (32). Similarly, movement within the tumour microenvironment may be altered by immune evasion strategies to prevent optimal antigen presentation and T cell motility, as discussed below. The extent of CXCL12's role in T cell motility within the BM parenchyma itself is still unclear.

In the setting of inflammation, lymph node egress is reduced and mediators including hyperthermia, IL-6 and ICAM-1, enhance T cell homing to the lymph node. Tumor necrosis factor-dependent production of CXCL9 and CXCL10 on HEV surfaces allows recruitment of $CXCR3^+$ effector T cells and NK cells, whilst CCL3 and CCL4 production in the paracortex mediates enhanced trafficking and multicellular encounters of $CCR5^+$ naïve and memory $CD8^+$ T cells with $CD4^+$ T cells and DCs (24).

T cell positioning in relation to antigen and inflammatory cues in secondary lymphoid organs is key in determining T cell phenotype. Expression of CXCR3 by central memory $CD8^+$ T cells is essential for efficient antiviral recall responses, due to re-localisation towards antigen (33). In $CD4^+$ T cells,

CXCR3 upregulation is associated with differentiation to a T_H1 phenotype, and amplification of interferon-γ (IFN-γ) dependent recruitment to peripheral sites of infection (34). As a transcriptional target of T-bet, CXCR3 expression has also been suggested to predispose both $CD4^{+|}$ and $CD8^+$ T cells towards an effector phenotype (35).

Another chemokine receptor, CCR5, is also known to play an important role in lymphocyte trafficking. In conjunction with ligands CCL3, CCL4 and CCL5 it has been implicated in GVHD and solid organ transplant rejection. CCR5 is known to be important for lymphocyte recruitment to GVHD target organs such as liver, lung and spleen. CCR5 blockade has been explored as a therapeutic option to prevent visceral acute GVHD with promising results. The finding that CXCR3 signaling may be an important resistance mechanism in this case highlights the overlap in chemokine function (36, 37).

Chemokines also play in central role in humoral immunity. Follicular dendritic cell networks direct B cell localisation in mature lymph nodes via CXCR5 and CXCL13, and in the spleen marginal zone B cells use CXCR7. Chemokine control over immune localisation is reinforced in tertiary lymphoid organs at sites of chronic inflammation. Inflammatory cytokines induce expression of lymphocyte-attractants CXCL13 and CCL19, and hence extend chemokine control over the range of immune cells in the periphery (26).

Inflammatory chemokines also direct migration of the innate immune system, via expression of CXCR2, CCR1 and CXCR1 on neutrophils, CXCR2 on monocytes, CCR1 and CCR3 on eosinophils and CXCR3 and CXCR6 on NK cells, amplifying subsequent binding to selectins and integrins and hence

promoting endothelial transmigration to the source of inflammation. Chemokines also have an important role in angiogenesis, primarily defined by presence of the ELR motif. Angiogenic, ELR-positive chemokines, such as CXCL1 and CXCL8/IL-8, act through CXCR2 whilst non-ELR chemokines, such as CXCL4, are angiostatic and act through CXCR3B. The exception is CXCL12, a non-ELR containing angiogenic chemokine, with relevance in physiological (38) and malignant settings (39).

CXCR4 plays an important role in orchestrating tissue regeneration following injury and inflammation. High mobility group box 1 (HMGB1) functions as a damage-associated molecular pattern, released on injury to trigger inflammation. HMGB1 has been shown to form a complex with CXCL12, inducing migration of muscle stem cells and regeneration via CXCR4 interactions (40). Furthermore, the transcription factor hypoxia-inducible factor 1a (HIF-1α) binds to the hypoxia-responsive element (HRE) on the CXCL12 promoter under conditions of low oxygen levels, promoting CXCL12 increase at the site of tissue injury. This homeostatic process is essential to damaged tissue repair and migration of progenitor cells, for example following bony fracture. This interaction can become corrupted because like many haematopoietic and non-haematopoietic tissue-committed stem/progenitor cells (TCSCs) expressing CXCR4 (41), cancer stem cells can use the same axis to egress from their tissue of origin. In much the same way as lymphocytes egress into lymph nodes, these cells can then adhere to metastatic niches using reciprocal CXCR4 interactions with integrins and selectins such as LFA-1, VLA-4 (very late activation antigen-4) and L-selectin (42, 43).

1.3 The CXCL12-CXCR4 axis: structure and signaling

A Japanese group, studying the relationship between the bone marrow microenvironment and the haematopoietic system first discovered the importance of CXCL12 (44). Interleukin 7 (IL-7) had been identified as a cytokine produced by bone marrow stromal cell lines which enhanced pre-B cell proliferation but although several lines of evidence pointed to the requirement for an as yet unidentified factor produced by the PA6-stromal line, none of the tested cytokines could induce proliferation (45). To address this issue, Takashi Nagasawa and co-workers first cultured stromal cells in the presence of haematopoietic cells separated by a membrane filter, proving the importance of a soluble factor in proliferation of B cell precursors. They subsequently isolated a complementary DNA (cDNA) clone encoding this growth-stimulating factor and initially named the molecule PBSF (pre-B-cell growth-stimulating factor). The amino acid sequence was soon found to be identical to the recently cloned stromal cell-derived factor 1α (SDF-1α, otherwise known as CXCL12). Direct contact between stromal and haematopoietic cells was important for cell survival, suggesting avenues for later work which unveiled the importance of CXCL12 in the context of the niche (44).

Further studies from the same group identified the CXCL12 receptor partner, CXCR4, by synthesizing conserved transmembrane proteins known to be present in previously identified chemokine receptor chains and using them as primers in reverse transcription PCR experiments from a CXCL12 responsive pre-B-cell clone. To their surprise, the identified seven-transmembrane

receptor corresponded almost exactly to a human HIV-1 entry coreceptor known as fusin (46). They went on to show that *Cxcl12* knockout was embryonic lethal in mice, inducing severe defects in B-cell progenitors, bone marrow myelopoiesis, cerebellar and cardiac defects (47). Other groups showed that CXCL12 could prevent HIV-1 infection of T cells (48).

The CXCL12-CXCR4 axis has since been implicated in many homeostatic processes including haematopoietic stem cell (HSC) homing and maintenance, B cell and plasma cell maintenance, embryogenesis, vascular, cardiac and neurological development, in addition to T cell trafficking (49-51). The same Japanese group confirmed that deletion of CXCR4 in adult mice severely impaired the numbers of HSCs, altered cell-cycle status and increased sensitivity to DNA damage, but had less effect upon mature progenitors. Importantly, most HSCs were in contact with a small population of reticular cells with long processes which are prominently associated with vascular niches and express large amounts of CXCL12 (52).

Defects induced in mice lacking CXCR4 are identical to those in CXCL12-deficient mice, confirming the importance of this pairing (50). Mice reconstituted with CXCR4-deficient fetal liver cells have reduced granulocytic cells in the bone marrow and elevated peripheral blood progenitors, suggesting therapeutic potential later exploited to mobilise stem cells for transplantation (53). These twin roles as growth promoter and chemoattractant highlight the unexplored therapeutic potential of this signaling axis, which will be discussed further in this thesis.

Recent studies have also highlighted unsuspected roles for CXCL12, such as a critical role in linking innate and adaptive immunity. Neutrophils have been shown to leave long-lasting trails of packaged CXCL12 to guide virus-specific $CD8^+$ T cell recruitment to infected organs (54). Tissue insults such as hypoxia, toxins or irradiation also act to increase local CXCL12 expression, improving recruitment of CXCR4-expressing stem cells to the microenvironment. These studies highlight pleiotropic roles in inflammation and immune surveillance, straddling the boundary between homeostatic and inflammatory chemokines (55-57).

CXCL12 therefore stands apart from its fellow CXC chemokines in a number of ways, not least its chromosomal location. Whilst most CXC chemokine genes are located on 4q21, CXCL12 is situated on the long arm of chromosome 10. Expression is tightly regulated, presumably due to its numerous critical roles; it is the only CXC chemokine to exhibit differential splicing with six different variants in humans and three variants in mice (58). The classical α and β variants bind CXCR4 with comparable affinity but the importance of the different tissue distribution and function of the alternative isoforms is unknown. The high degree of sequence and amino acid homology between humans and evolutionarily distinct species such as frogs and zebrafish confirms its primordial biological importance (59).

CXCL12 initiates signal transduction by binding to the membrane-spanning G protein-coupled receptor and activating intracellular heterotrimeric G protein subunits **(**α, β, and γ). GPCRs are the largest family of membrane proteins, mediating most hormonal and neurological signals. CXCR4 is a member of the rhodopsin family, characterised by significant sequence homology and

implying shared activation mechanisms. Recent crystal structures of CXCR4 in complex with antagonists have however suggested it has a different shape and binding site location to other GPCRs such as rhodopsin, the β_2-adrenergic receptor and the A_{2A} adenosine receptor (60).

Intracellular signalling

Activation of the G protein heterotrimer triggers release of bound guanine diphosphate (GDP), replacement by guanine triphosphate (GTP) and dissociation into α and βγ subunits. Gα itself consists of 4 families: $G\alpha_i$, $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12}$. $G\alpha_i$ inhibits adenyl cyclase, while $G\alpha_s$ stimulates this pathway, resulting in conversion of ATP to the second messenger cyclic adenosine monophosphate (cAMP). $G\alpha_q$ acts on phospholipase C (PLC) to hydrolyse phosphatidylinositol 4,5-biphosphate (PIP2) to generate inositol triphosphate (IP3) and diacyl glycerol (DAG), which increase intracellular calcium concentrations and activate multiple protein kinases including the mitogen-activated protein kinase (MAPK) pathway inducing chemotaxis. $G\alpha_{12}$ also regulates chemotaxis via the Rho and Ras families of small GTPases (61). The majority of G-protein coupled pathways involved in CXCR4 are thought to be $G\alpha_i$ dependent as they are inhibited by pertussis toxin through ADP-ribosylation in the cytoplasm (62) while the Gβγ subunit can also trigger PLC activation and calcium flux. Both $G\alpha_i$ and Gβγ activate the phosphatidylinositol 3-kinase (PI3K) pathway to trigger chemotaxis independently, or mediate transcription of cell survival genes through the serine-threonine kinase Akt (61) (Figure 1-1).

CXCL12 also triggers receptor desensitisation, via G-protein uncoupling, phosphorylation of the CXCR4 cytoplasmic tail by protein kinase C and GPCR-kinases and subsequent β-arrestin interactions. These β-arrestins then target CXCR4 for endocytosis in clathrin-coated pits, and some arrestin pathways are also linked to further p38 and ERK kinase signaling and chemotaxis (62). Further G protein-independent signalling is mediated by activation of the JAK/STAT family of transcription factors (63). In summary, CXCR4 activation triggers multiple downstream intracellular signaling pathways variously resulting in chemotaxis, cell survival, proliferation, gene transcription and increased intracellular calcium (64) (Figure 1-1).

CXCR4 is expressed on an enormous variety of cell types, ranging from haematopoietic progenitors to peripheral blood B and naïve/activated T cells, plasma cells, monocytes, NK and dendritic cells, mast cells, vascular smooth muscle cells, endothelial cells, microglia, neurons, astrocytes and some epithelial cells, as well as stromal cells in organs such as lung, bone marrow and small bowel (50, 65-73). The complexity of the CXCR4 system is highlighted by the variety and nature of alternative ligands and antagonists. Other CXCR4 ligands include the HIV envelope glycoprotein gp120, extracellular ubiquitin and macrophage inhibitory factor (MIF), which also mediate T cell chemotaxis and arrest, and trefoil factor 2 (74) and human beta defensin protein 3 (75), which do not. The extent and importance of these alternative pathways is still unknown, as is the role of ACKR3 in CXCL12 signaling. ACKR3 has over 10-fold higher affinity for CXCL12 compared to CXCR4, and is thought to mediate G protein-independent signaling, although this is controversial (73). Furthermore, heterodimerization of ACKR3 with

CXCR4 has been shown to cause β-arrestin-mediated downregulation of $G\alpha_i$ signaling, inducing activation of MAP kinases ERK1/2, p38 and SAPK in T cells (17, 76). Further evidence demonstrating that ACKR3 function is not redundant includes its ability to stimulate neural progenitor cell proliferation in response to CXCL12, in contrast to CXCR4 (77). ACKR3 also has an essential role in homing and migration of normal progenitors and acute myeloid leukaemia (AML) cells to cellular niches (78). CXCL12 signaling is therefore cell-type and context-specific. In some tissues, heterodimerization is required, or CXCR7 substitutes for CXCR4 to mediate crucial signaling, further highlighting the unique characteristics of this pathway (79).

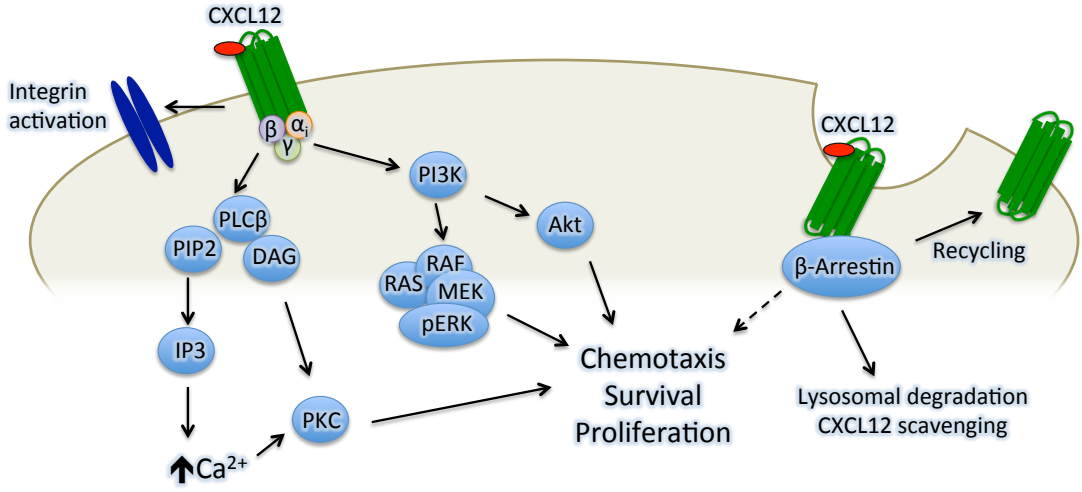


Figure 1-1 CXCR4-CXCL12 Signalling Pathways
CXCL12 binding to CXCR4 activates a variety of intracellular pathways resulting in diverse outcomes including cell migration, integrin activation and transcriptional activation of survival and proliferation pathways. β-arrestin pathways can result in receptor ubiquitination and degradation or recycling, but may have other downstream effects (dotted line).

1.4 Regulation of CXCR4 and CXCL12

Understanding the tight regulation of both gene and protein expression is important to decipher the role of CXCR4 in health and disease. The major transcription factor positively regulating CXCR4 expression is Nuclear Respiratory Factor-1 (NRF-1), acting on the promoter, with additional effects from the SP-1 transcription factor. The opposing negative regulatory factor is thought to be Yin-Yang 1 (62, 80). CXCR4 expression and signaling activation is upregulated by a host of molecules including calcium, cyclic AMP, IL-2, IL-4, IL-7, IL-10, IL-15, transforming growth factor beta 1 (TGFβ-1), CD3 and CD28 costimulation, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). In contrast, TCR activation, CXCL12 signalling, tumour necrosis factor-α (TNF-α), IFN-γ and IL-1β all reduce CXCR4 expression (81-85).

The CXCR4 protein is further modified by post-translational glycosylation and tyrosine sulphation, both of which alter CXCL12 binding. Differential hetero- or homodimerisation and even tetramer formation of CXCR4 with other receptors such as CCR2, CD4 and CD8, has been proposed as a further level of regulation to alter signaling (86-88). Various extracellular molecules also modify CXCL12 binding. For example, during inflammatory degranulation, cathepsin G and neutrophil elastase cleave five N-terminal residues on CXCL12, yielding inactive forms of the chemokine (6-67) (89). Disruption of CXCL12 signaling appears to have a dominant role in mobilization of HSCs following administration of granulocyte colony stimulating factor (GCSF), a

drug that is used in haematopoietic stem cell transplantation (HSCT) for a variety of diseases. The precise mechanism underpinning its biological effect is still unclear, but G-CSF treatment leads to reduction in BM CXCL12 mRNA and protein levels, and reduced CXCR4 expression on mobilised HSCs, and is likely to relate to the effect of neutrophil-derived proteases suppressing interactions between stroma and HSCs (90).

Numerous other pathways modulate CXCL12/CXCR4 signaling, notably CD26 (dipeptidylpeptidase 4, DPPIV). This membrane-bound peptidase cleaves dipeptides from N-termini of certain molecules, including CXCL12. CD26 is expressed on the surface of a large variety of haematopoietic cells, including HSCs, progenitors, activated T cells, stimulated B and NK cells, and also fibroblasts, endothelial and epithelial cells. CD26 is also present in a soluble active form in plasma. Truncated inactive CXCL12 acts as an antagonist, blocking chemotaxis of full-length CXCL12. CD26 inhibitors, such as diprotin A, are able to enhance chemotaxis of bone marrow progenitors in response to CXCL12. Interestingly CD26 inhibition interferes with GCSF-induced progenitor mobilization, suggesting that CD26 is involved in the mechanism of action (91, 92).

Exploitation of the DDP4 axis has been suggested as a potentially tractable route to improve the efficiency of HSC mobilization and transplantation. Several selective DPPIV inhibitors are safe and approved in medicine for other indications such as type 2 diabetes. Sitagliptin has been trialed with some success in an attempt to maintain higher levels of active CXCL12 and enhance engraftment in cord blood transplants (93, 94). Encouraging yet inconclusive results suggest that the ability of DPPIV to modify colony

stimulating factors and other chemokines such as CXCL10 may have additive effects upon HSCs and lymphocyte trafficking, in the tumour and bone marrow environment (95, 96).

To add to the variety of factors interacting with CXCL12, GAGs such as heparin sulfate interact with CXCL12 polymers to aid chemokine immobilization of the chemokine, forming gradients for leukocyte migration (97, 98). Alternative strategies can also prime the CXCR4 response in HSCs to lower levels of CXCL12, for example via platelet-derived micro-particles or leucopheresis products containing adhesion and coagulation-related molecules, possibly through incorporation of membrane CXCR4 into lipid rafts (99, 100).

1.5 Chemokines and receptors in disease

With the notable exception of CXCR4, the majority of homeostatic chemokine receptors do not play a major role in disease. Inflammatory chemokines are by contrast heavily implicated in a wide variety of pathological processes. For example, CCR5 is implicated in the development of multiple sclerosis (101), rheumatoid arthritis (102), inflammatory bowel disease (103) and Acquired Immuno Deficient Syndrome (AIDS) (104), while neutrophil receptors such as CXCR1 and CXCR2 are implicated in sepsis and atherosclerosis amongst others (105-107) . The profound effect of mutations in each of these chemokines highlights the importance of correctly functioning signaling pathways.

In the example of CXCR4, a heterozygous truncation mutation in the cytoplasmic tail results in enhanced leukocyte chemotaxis to CXCL12 and the clinical WHIM syndrome (warts, hypogammaglobulinemia, infections and myelokathexis). This causes neutropenia with excessive retention of mature neutrophils in the BM and impaired receptor desensitization (108). WHIM patients have responded to low dose treatment with the CXCR4 antagonist AMD3100 (Plerixafor), highlighting the potential susceptibility to pharmacological modification of this axis. CXCR4 has also been implicated in various inflammatory and autoimmune disorders including inflammatory bowel disease, rheumatoid arthritis and lupus (109, 110), where the degree of renal CXCL12 expression correlates with severity of lupus nephritis, further highlighting the double-edged nature of chemokine signaling.

1.6 Chemokines in the cancer microenvironment

Of relevance to this thesis, CXCR4 is the most common chemokine receptor expressed on cancer cells, having been identified in at least 23 different cancers of diverse origin (111). Cancer cell populations have co-opted the CXCR4-CXCL12 axis to enhance adhesion, angiogenesis and metastasis to distant organs. CXCR4 is the key signaling axis mediating metastasis in breast cancer to organ sites such as lung, brain, liver and bone marrow; these organs express the highest levels of CXCL12 in the body, and expression is upregulated under hypoxic conditions, acting to promote survival and invasion. The CXCR4/CXCL12 axis promotes an invasive metastatic phenotype in

ovarian and medullary thyroid cancer (112-114), and high CXCL12 levels are associated with worse survival in ovarian cancer amongst many others (115). The nuclear factor-κB (NF-κB) transcription factor binds the CXCR4 promoter to promote dysregulation and chemotherapy resistance in a host of cancers (116, 117) and CXCR4 expression has been suggested as a marker of 'stemness' in solid tumours (118, 119).

In the haematological setting, CXCR4 occupies a similarly central position. Somatic, WHIM-like CXCR4 mutations are present in over 25% of patients with the B-cell malignancy, Waldenstrom's macroglobulinemia. These mutations activate the AKT and ERK/MAPK signaling pathways, are associated with higher BM involvement and resistance to novel B-cell receptor targeted agents such as ibrutinib (120, 121). Nearly half of all myelomas over-express CXCR4, which is implicated in cell adhesion-mediated drug resistance (122). B-cell chronic lymphocytic leukaemia (CLL) cells express higher levels of CXCR4 than normal B cells, in association with increased chemotactic responses to CXCL12 secreted by stroma or protective nurse-like cells (123, 124). CXCR4 expression in acute myeloid leukaemia (AML) is an independent predictor of disease relapse and has a dismal effect upon overall survival of similar magnitude to that of unfavourable cytogenetics (125). High CXCR4 expression in B-cell acute lymphoblastic leukaemia (ALL) predicts extramedullary organ infiltration and worse survival (126, 127) and has recently been found to be essential for leukaemia-initiating cell activity in T-ALL (128). As in the context of normal T cells, CXCR4 triggering in T-ALL leads to integrin receptor activation, allowing migration across vascular beds. Interestingly, ACKR3 is also overexpressed by both ALL and AML cells; it

contributes to CXCL12-induced T-ALL migration, possibly by preventing CXCR4 downregulation, suggesting a potential leukaemic resistance mechanism (78, 129). In the context of standard therapy approaches, chemotherapy has been shown to induce CXCR4 upregulation in AML cell lines and subsequent stromal protection from apoptosis (130). Stromal CXCL12 promotes an enhanced energy supply (glycolytic shift) via the mammalian target of rapamycin (mTOR) signaling axis in AML cells (131, 132). Further effects follow from CXCR4-mediated downregulation of the micro RNA, let-7a, which drives chemoresistance. Similarly, studies in chronic myeloid leukaemia (CML) have shown that an unexpected effect of BCR-ABL kinase inhibition by imatinib is to trigger CXCR4-dependent migration to BM stroma, leading to cell cycle arrest and drug resistance (133).

1.7 Antagonizing the CXCR4/CXCL12 signaling pathway

Given the profound importance of CXCR4 in a host of disease processes, attempts at antagonising this pathway are of great research interest. AMD3100 (Plerixafor), a bicyclam compound, was initially developed as an inhibitor of human immunodeficiency virus (HIV) replication in 1994 (134, 135). The surprising lack of activity against simian immunodeficiency virus (SIV) was explained three years later when it became clear that the mode of action was through direct inhibition of T-cell tropic strains of HIV (not present in SIV) interacting with CXCR4 after binding to the CD4 receptor on T cells (136, 137). Plerixafor is able to selectively and reversible antagonise chemotaxis to

CXCL12, resulting in complete blockade of the normal calcium flux response to CXCR4 signaling. Subsequent clinical trials in HIV patients noted a rapid dose-dependent increase in peripheral blood white blood cell counts, in particular those carrying the stem cell marker $CD34^+$ (138).

In combination with the established stem cell mobilizing agent, GCSF, Plerixafor induces a tripling of selective stem cell mobilization and has become a standard of care for people failing standard autologous mobilization regimes prior to transplantation for lymphoma or myeloma. Interestingly, Plerixafor-related stem cell mobilization may occur without more tumour cell contamination than occurs with GCSF alone, as long-term survival outcomes after autologous transplantation are equivalent (139). Direct comparison is difficult because the stem cell product mobilised by Plerixafor is qualitatively different compared with GCSF. Plerixafor has been suggested to mobilise more of a primitive $CD34^+/CD38^-$ stem cell subpopulation, as well as increased number of $CD4^+$ and $CD8^+$ T cells, NK cells and plasmacytoid DCs, independent of stem cell yield (140). Other studies have suggested GCSF alone mobilises more of the most primitive multipotent progenitors ($CD34^+CD38^{low}CD133^+CD45RA^-$) whilst combination treatment with GCSF and Plerixafor yields more mature $CD34^+$ progenitors and differentiated cells such as late granulocyte-monocyte progenitors (GMPs) and $CD8^+$ T, B and NK cells (141); this latter study is difficult to interpret because only poor mobilisers received combination therapy. Another recent study has shown that intravenous Plerixafor preferentially mobilises more pre-plasmacytoid DCs with a putatively favourable immuno-regulatory phenotype, correlating

with low CMV viraemia and GVHD rates post allogeneic stem cell transplant (142).

Recent studies have suggested that the gene expression profile of Plerixafor-mobilised T cells differs from that characteristic of T cells mobilised by GCSF. Plerixafor-mobilised human T cells had similar phenotype and gene expression levels to non-mobilised T cells, whereas GCSF-mobilised cells had significant reduction in $CD62L^+$ $CD4^+$ and $CD8^+$ T cells, and lower proportions of $CD27^+$ $CD8^+$ T cells (143). CD27 is a costimulatory molecule involved in augmented T cell activation and required for the development and maintenance of long-term memory (144). Whilst CD62L shedding post GCSF-administration may be a temporary phenomenon (143), the association of Plerixafor-mobilised grafts with increased GVHD relative to GCSF-mobilised grafts in murine transplant models (145) suggests that CXCR4 antagonism may mobilise a more potent memory T cell subset from putative BM niches. The picture is complicated by GCSF-mobilisation inducing a skewed T helper 2 and regulatory $CD4^+$ T cell phenotype (146, 147), which may affect GVHD rates.

Several groups have tested the biological activity of CXCR4 inhibitors in synergy with chemotherapy or targeted kinase inhibition in acute and chronic leukaemias (148, 149). Theoretically, inducing mobilization of leukaemic stem cells could sensitize them to chemotherapy outside a protective BM niche; however clinical responses have thus far failed to yield dramatic benefits (150, 151). More potent agents including an apoptosis-inducing humanised anti-CXCR4 antibody (ulocuplumab) are in clinical development (152). In murine models, Plerixafor has also been shown to inhibit collagen-induced arthritis

(153), medulloblastoma and glioblastoma (154), promote blood flow restoration in diabetic ischaemia (155) and enhance survival post West Nile virus via enhanced central nervous system (CNS) $CD8^+$ T cell homing (156). Intriguingly, CXCR4 blockade has also been reported to reduce the proportion of exhausted $CD4^+$ T cells and improve survival in murine polymicrobial sepsis (157). The authors speculate that this may be due to replenishment of exhausted peripheral T cells from bone marrow populations of PD-1^{low} T cells. These results would be consistent with mobilisation from the BM of a more potent T cell subset.

1.8 Malignancy and immune evasion

Although initiating events in development of malignancy often involve sustaining proliferative signaling and resisting cell death, further mutations and epigenetic modifications are usually necessary to create successful malignant phenotypes, comprising alteration of metabolic pathways, evasion of immune destruction and metastasis (158). This process takes place over a prolonged period of time associated with multiple interactions with the tumour microenvironment and the immune system. The idea that the immune system could control the growth of pre-malignant cells was first given voice in 1909 by Ehrlich (159), and later developed by Burnet and Thomas (160, 161). Immunosurveillance remained a hypothesis, despite evidence of increased tumour rates in immuno-suppressed transplant patients, until the development of knockout mice deficient in immune effector cells in recent decades.

Numerous studies have now demonstrated that T cells can recognize neoantigens, successfully infiltrate and induce long-term responses in diverse tumours such as melanoma, colon and ovarian carcinoma (154, 162, 163).

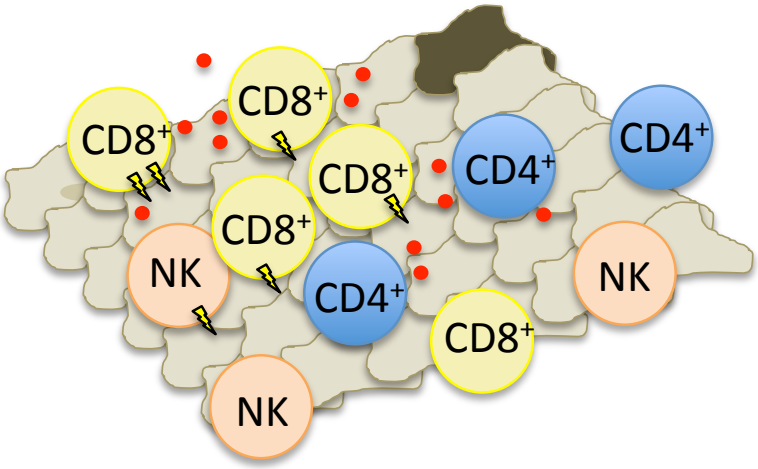
The genomic instability present in most cancers dictates that as cancer cells acquire mutations in protein-coding genes or epigenetic alterations, they express tumour-specific (TSAs) or tumour-associated antigens (TAAs). Some of these antigens, either neoantigens or embryonic, are expected to be immunogenic due to recognition of the non-self (or 'non-adult self') peptides in association with major histocompatibility complexes (MHC) on the cancer cell surface by cytotoxic T lymphocytes.

This is evidenced by variation in the degree of immunogenicity in tumours based upon mutation frequency. For example, melanomas as the prototypical immune-sensitive tumour are highly mutated due to the effects of UV light, and have been shown to spontaneously regress in association with clonal expansion of T cells (164). Similarly microsatellite instability (MSI) in colorectal cancer, which involves DNA mismatch repair defects and hence high levels of genomic instability, is strongly predictive of activated cytotoxic T cell infiltration, reduced metastases and better survival than MSI negative cases (165). Renal and bladder cancers, despite a relative paucity of somatic mutations, are also responsive to immunotherapy, suggesting the existence of multiple factors dictating the quality of the antigenic stimulus and corresponding immune response (166).

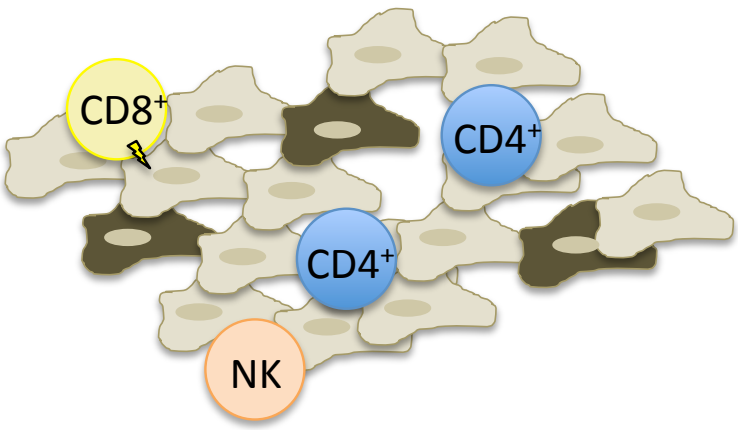
In response to the apparent failure of the immune system to control cancerous growth in individuals first presenting with cancer, the immunosurveillance

hypothesis has been refined by acknowledging a complementary role in selecting for tumours with decreased immunogenicity. This process of immunoediting is conceived as three sequential phases: elimination, equilibrium and escape (167). In the first phase, malignant growth induces inflammatory signals recruiting first the innate immune system (macrophages, NKT and NK cells, γδ T cells and dendritic cells) and subsequently an adaptive response by means of IFN-γ, CXCL9, CXCL10, CXCL11 and IL-12 to destroy antigen-bearing tumour cells. In the equilibrium phase, the inherent genetic instability of tumours that have not been destroyed induces multiple escape variants, some of which are able to persist and hamper immune detection, although the majority of tumour cells are still undergoing detection and a degree of destruction. As this phase evolves, the selection pressure of the immune system increasingly enables genetic and epigenetic alterations in the tumour and microenvironment to downregulate the immune response, leading to malignant growth.

Elimination



Equilibrium



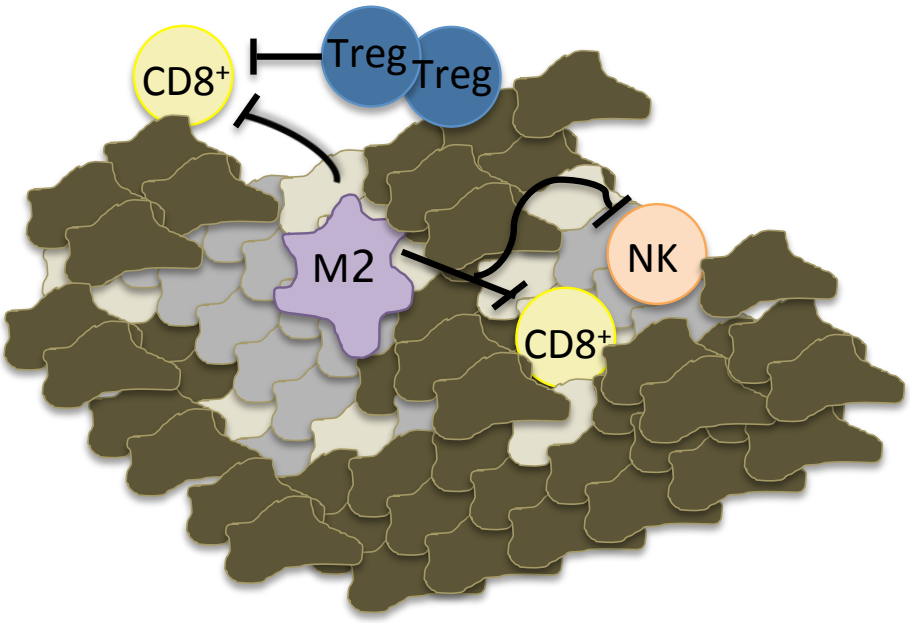


Figure 1-2 Cancer immunoediting.

The three phases by which the immune system sculpts malignancy to evade cytotoxic attack. Elimination involves chemokines and cytokine (red circles) recruitment of immune effectors such as $CD4^+$ and $CD8^+$ T cells, NK and NKT cells to engage in cytotoxic destruction (yellow lightning bolt) of tumour (beige). As this Darwinian process proceeds to dynamic equilibrium, the immune response promotes the increasing prevalence of mutated tumour variants (brown and grey) more capable of withstanding an immune response, until these variants become the predominant cell type in the Escape phase, suppressive cell types such as regulatory T cells (T_{reg}) and tumour-associated macrophages (M2) suppress the cytotoxic response, and the tumour is able to expand unchecked.

Evolutionary theory predicts that different tumours will employ a large array of different strategies to induce a suppressive TME (168). This may occur through blockade of physical T cell recruitment to the tumour, for example cancer-associated fibroblasts (CAFs) coat pancreatic carcinoma cells with CXCL12 to exclude T cells (169) or nitration of the chemokine CCL2 by myeloid-derived suppressor cells (MDSCs) in prostate and colon cancer (170). Tumour-associated endothelial cells can be induced to express Fas ligand (FasL) signals to preferentially recruit $Foxp3^+$ regulatory T cells (T_{reg} cells) and myeloid cells rather than T effectors (171). T_{reg} cells suppress anti-cancer immunity through several distinct mechanisms including CTLA4-mediated inhibition of antigen-presenting cells (APCs), consumption of IL-2 and secretion of inhibitory molecules including IL-10 and TGF-β.

A recent study has elegantly highlighted the intimate connection between homeostatic immunoregulatory processes and their corruption in the tumour microenvironment. The enhanced sensitivity of T_{reg} cells to oxidative stress suggests their utility in limiting excessive reactive oxygen species generated by neutrophils in the setting of infectious and inflammatory stimuli. Apoptotic T_{reg} cells therefore release ATP, which is converted to immunosuppressive adenosine to dampen down harmful immune-related destruction of normal or malignant tissue once the initial stimulus has been cleared. This pathway is co-opted by ovarian cancer when free oxygen species in the tumour microenvironment induce T_{reg} cell apoptosis, triggering a suppressive cascade to nullify effective T and APC responses (172).

Other mechanisms of immune suppression include hypoxia-triggered upregulation of programmed cell death protein ligand 1 (PD-L1) on

myelomonocytic cells (173), tumour and myeloid cell production of immunomodulatory enzymes such as indoleamine 2,3 dioxygenase (IDO) (174, 175) and B cells reprogramming macrophages to a tumour-associated phenotype (176). Myeloid-derived suppressor cells can themselves induce T_{reg} cell production, produce TGF-β and deplete arginine and cysteine or nitrosylate the T cell receptor (TCR) to inhibit T cell function (177). Finally, breast and lung cancer cells can directly trigger activation of the nucleotide-binding oligomerization domain-, leucine-rich repeat- and pyrin domain-containing 3 (NLRP3) inflammasome and IL-1β to induce $CD4^+$ memory T cells to secrete the cancer-promoting cytokine IL-22.

1.9 T cell memory

> *'The same man was never attacked twice'*
> Thucydides recounting the plague of Athens, 430BC (178)

Immune memory as first observed by Thucydides is defined as the ability of the immune system to respond more effectively to a secondary encounter with non-self antigen (179). The persistence of long term memory for many years following initial exposure, for example in yellow fever vaccination, suggests the facility for a long-lived population of antigen-specific lymphocytes that re-expand upon secondary encounter to drive a more effective response (180). An anti-tumour memory T cell response thus represents a potent long-term solution to the thorny issue of preventing both disease relapse and chemotherapy-resistant clonal evolution (181).

Memory T cells demonstrate great functional and phenotypic heterogeneity, resulting from the quality of the initial antigenic stimulus and antigen persistence, coupled to environmental signals including the balance of co-stimulatory and co-inhibitory molecules, homeostatic cytokines and stromal inputs (182-184). The ontogeny and lineage relationship between different subsets is still a matter of ongoing debate and at least three models of memory formation have been put forward, each supported by differing strands of experimental evidence (185).

Linear differentiation model

This model proposes that naïve T cells differentiate directly into effector T cells (T^{EFF}) after priming. The majority of T^{EFF} ($KLRG1^{HIGH}CD127^{LOW}$ short-lived effector cells, SLECs) die after pathogen clearance but a subset of effectors (memory precursors, MPECs) develop further into $CCR7^{-}$ $CD62L^{-}$ effector memory T cells (T^{EM}) and then central memory T cells (T^{CM}). T^{EM} cannot home to lymphoid tissues or recapitulate earlier T cell subsets, while T^{CM} can self-renew in the absence of antigen through homeostatic proliferation. This model is based upon studies showing that T^{CM} become the predominant subset after transfer of a predominantly T^{EM} subset (186). New light has been shed upon these findings by recent data from epigenetic analyses. Recent reports suggest that MPECs can acquire then erase repressive methylation programs, allowing them to re-express critical naïve-associated genes such as *Sell* (coding for CD62L), *Ccr7* and *Bcl2* (187). Intriguingly, long-lived memory cells appear to retain an 'epigenetic memory'

of transitioning through an activated stage, delineated by persisting demethylated CpG sites near loci for granzyme B and perforin. This effector-like epigenetic landscape is very different from the methylation pattern seen in naïve T cells. Unlike effector cells, long-lived memory cells do not express either granzyme B or perforin, and have a naïve-like transcriptome, but the transcriptional permissiveness of their open chromatin suggests they are poised to rapidly acquire effector functions if needed (187, 188).

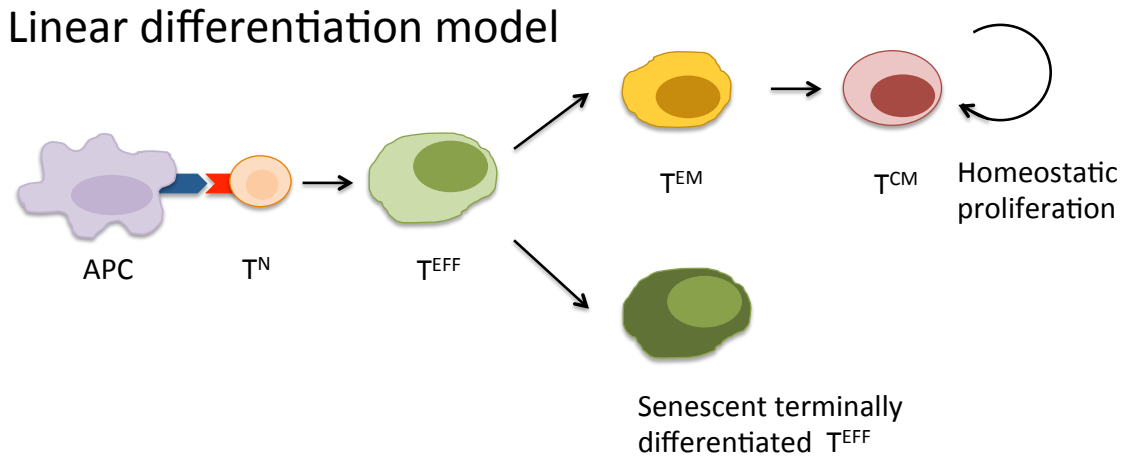
Bifurcative model

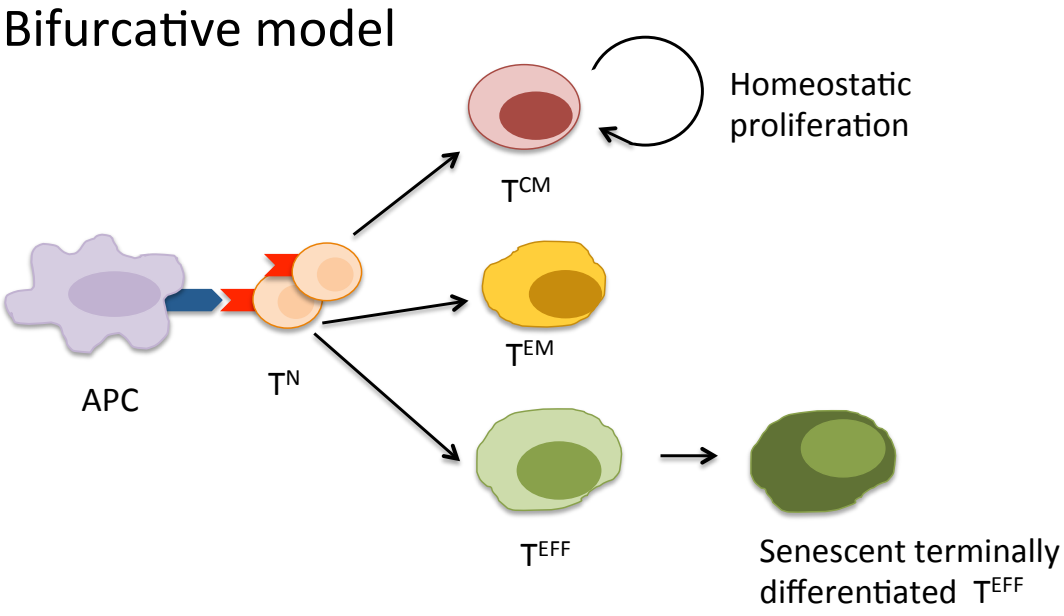
The bifurcative model proposes a single naïve T cell can give rise to differentially fated daughter cells by means of an important evolutionarily conserved mechanism known as asymmetric cell division. The positioning of daughter T cells proximal or distal to the APC at first cell division results in unequal distribution of key molecules mediating cell fate, including CD8, CD25, the transcription factor T-bet and the master regulator of cell growth mammalian target of rapamycin complex 1 (mTORC1) (189-191).

Progressive differentiation model

Also known as the signal-strength model, this delineates a clear pathway from naïve to stem cell memory (T^{SCM}), central memory, then effector memory and effector cells (192). The greater the magnitude of the input signal, the further down the pathway the cells progress, losing self-renewal capacity and anti-tumour efficacy as they differentiate. Increasing evidence supports this model,

including telomere length measurements (193), mass cytometric analysis of phenotypic markers such as co-stimulatory molecules CD27 and CD28 (194, 195), gene-expression profiling (196-198) and in vitro analyses of self-renewal and differentiation (198, 199).





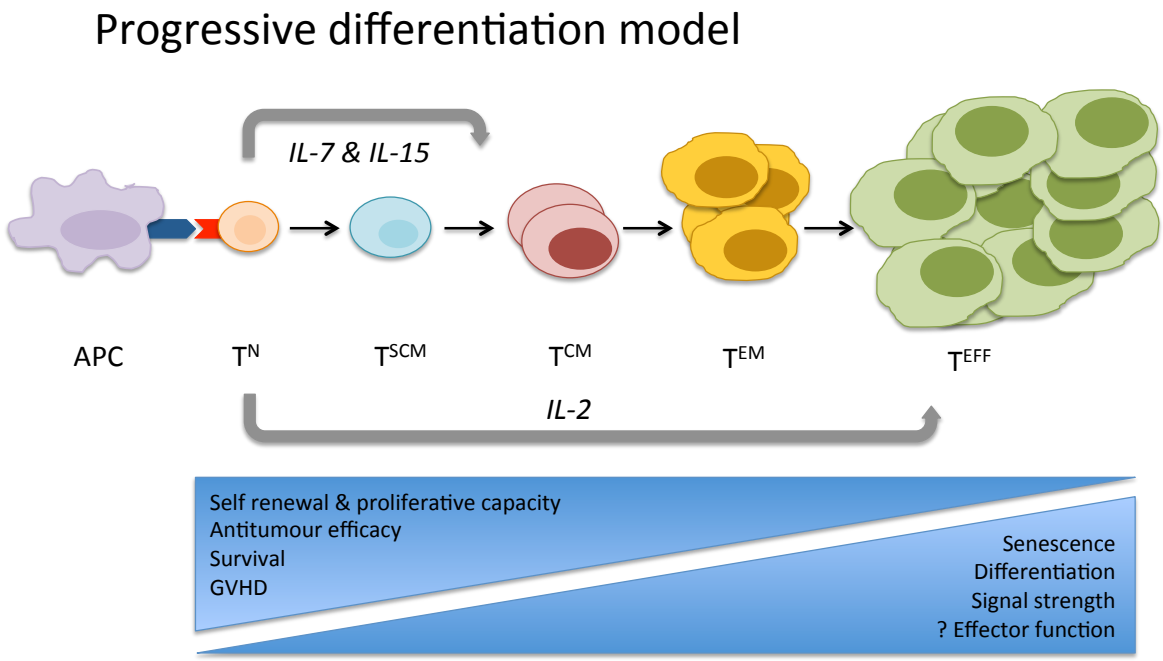


Figure 1-3 Models of T cell memory formation.
The linear differentiation model proposes that naïve cells (T^{N}) differentiate directly into effector cells (T^{EFF}) after interaction with antigen-presenting cells (APC). Effector cells either become terminally differentiated short-lived T cells, or survive to differentiate into effector memory (T^{EM}) and then T^{CM}. The bifurcative model proposes that asymmetric division of amino acids and other key determinants at time of priming determines cell fate, such that one T cell can give rise to two daughter cells: the distal daughter cell gives rise to T^{CM}, while the proximal cell develops into T^{EM} or T^{EFF}. The linear differentiation or signal-strength model proposes that T cell fate is determined by the strength of interaction with the APC and exposure to IL-7 and IL-15, such that weak interactions given rise to memory stem cells (T^{SCM}) and T^{CM}, which possess superior antitumour efficacy due to 'stemness' properties of self-renewal and multi-potency. Stronger T^{N}-APC interactions and IL-2 give rise to more differentiated T^{EM} and T^{EFF}.

These models represent a simplification and elements of these models overlap. In particular, the bifurcative model could be consistent with findings from the other two models. Other data using mice with Cre-mediated

expression of yellow fluorescent protein (EYFP) upon transcription of the gene for granzyme B (*Gzmb*) note that $CD8^+$ $CD62L^{high}$ cells remain present throughout the primary response to influenza infection and express *GzmB* (200) These data argue for a modification of the linear differentiation model and to some extent the bifurcative model in that T^{CM} do not develop from effector T cells, but as discussed above elaborate effector functions themselves through epigenetic modifications.

As well as great phenotypic diversity, and variation depending upon the antigen itself, there may well be diversity in mechanisms of memory formation encompassing phenotypically diverse memory populations evolutionarily adapted for cooperative roles in host defence. For example, tissue-resident memory T cells (T^{RM}) undergo no recirculation but function as important organ-specific sentinels at mucosal borders (201). A recent study showed that induced deletion of a transcriptional repressor gene *Id2* was able to transform $KLRG1^{HIGH}$ terminal effector/effector-memory $CD8^+$ T cells into a $KLRG1^{LOW}$ memory-like population, suggesting that the dogma of terminal programming may need to be revised to incorporate some flexibility of memory status, dependent upon sustained transcription factor regulation of target genes (202). Further elucidation of the complexities of memory fate specification awaits studies combining large-scale single-cell analysis with comprehensive in vivo fate mapping technologies. Much of the debate over ontology may be a semantic issue relating to experimental models and limited tools with which to delineate cell status. A more holistic definition of memory subsets examining self-renewal, homing and the epigenetic landscape in toto may resolve these issues.

The role of chemokines in T cell memory

In facilitating the interplay between innate and adaptive arms of the immune system chemokines play an important role in the development of immunological memory. CCR4 and CCR5 are important for naïve $CD8^+$ T cell interactions with DCs and $CD4^+$ T cells, and in turn for enhancing the quality and persistence of the memory $CD8^+$ T cell response. Inflammatory chemokine receptors such as CXCR3 and CCR5 also have important effects upon memory fate. CCR5 accelerates effector memory T cell recruitment to the airways early following viral challenge-induced inflammation (203) while the absence of CXCR3 enhances generation of memory cells at the expense of effector cells (35, 204). CXCR3 positions T cells in the marginal zone, exposed to high levels of antigen on APCs and promoting effector differentiation for control of acute infection such as influenza, while CCR10 and CXCR6 have been suggested to promote tissue-resident memory $CD8^+$ T cells formation (T^{RM}) (205). The role of CXCR4 in memory T cells is discussed separately in 1.11 below.

Memory subsets

T^{CM} and T^{EM} possess contrasting homing properties in viral infection models and can be differentiated by surface phenotype, being respectively $CD62L^{high}CCR7^{+}CD27^{high}$ and $CD62L^{low}CCR7^{-}CD27^{low/int}$. T^{CM} have a gene expression profile closer to naïve T cells, patrol lymph nodes, are more responsive to CCL19 and CCL21, but are present in equal quantities to T^{EM} in spleen and BM. In contrast T^{EM} express receptors for inflammatory

chemokines, preferentially patrol inflamed nonlymphoid organs and have a more senescent replicative history with impaired expansion potential (198, 206). All of the models of memory described above conclude that T^{CM} display greater capacity for accumulation at the location of antigen-exposure, self-renewal and anti-tumour efficacy compared to effector memory T cells (4). Consistent with preclinical models, the proportion of T^{CM} in the infused product corresponds very strongly with long-term persistence of CAR-T cells and improved outcomes in trials of patients with solid malignancies (207).

The key properties of immunological memory are thought to rest upon the capacity of long-lived lymphocytes to self-renew and generate effector lymphocytes in response to antigenic challenge (208). A population designated T stem cell memory (T^{SCM}), displaying a naïve-like $CD45RO^-$ $CD62L^+$ $CD45RA^+$ phenotype but superior antitumour responses to T^{CM} has now been described in humans, mice and non-human primates (198). Stem and central memory $CD8^+$ T cells share a transcriptional program and key properties of self-renewal and multi-potency with long-term haematopoietic stem cells (209). They express a common set of markers including CD62L, CCR7, IL-2/15Rβ, Sca-1 (in mice) and BCL2 and require IL-15 (210, 211). The limitations of stem memory $CD8^+$ T cells relate to difficulties identifying them in several models and isolating them for clinical use (5). Autologous T^{SCM} are preferentially depleted by commonly used conditioning chemotherapy such as cyclophosphamide and cytarabine, in contrast to preserved T^{CM} (212). This issue is particularly important given the limited availability of early memory T cells for autologous adoptive cell therapy (ACT) in older patients with lymphoma and solid tumours (212). Pre-transplant

depletion of $CD44^{low}CD8^{+}$ T cells has been shown to abrogate GVHD (213) suggesting an association with persistent GVHD that is not seen with T^{CM} (210). In addition, serial single cell transfer fate mapping experiments have shown adult tissue stem cells within the T^{CM} compartment, such that the tiny numbers of tertiary T^{CM} progeny from a single primary T^{CM} are able to reconstitute full antigen-specific immunocompetence in immunocompromised hosts (5).

A large number of factors are involved in promoting T cell memory. Initial formation of the immune synapse involves adhesion molecules stabilizing the initial structure then the mature immunological synapse forms as a tight cluster of T cell receptors surrounded by a ring of adhesion molecules. This allows transport of ligands into the central cluster to form stable long-lasting interactions (214, 215). Naïve T cells are then thought to require further signals to be primed efficiently, including a costimulatory signal 2 from T cell CD28 binding to CD80/CD86 on APCs become fully activated. This crucial link between innate and adaptive immunity is enabled by inflammatory cytokine and toll-like receptor (TLR) signaling upregulating CD80 and CD86 on DCs.

CD28 costimulation increases production of the transcription factors AP-1 and NF-κB, increases transcription of IL-2 and stabilizes IL-2 mRNA, resulting in T cell proliferation (216, 217). In the absence of CD28, little IL-2 is produced and hence activated cells die or become anergic (218). Activated $CD8^{+}$ T cells primarily use aerobic glycolysis for their bioenergetic requirements, while the dominant process in naïve and memory T cells is oxidative phosphorylation of pyruvate and fatty acids (219). CD28 has also been shown to promote

expression of carnitine palmitoyltransferase 1a (Cpt1a), facilitating mitochondrial fatty acid oxidation in memory cells (220).

The fate of activated T cells varies according to the strength of the activating signal and other local environmental signals, known as signal 3. These comprise a number of different cytokine and chemokine combinations often initially secreted by antigen-presenting cells, for example IFN-γ and IL-12 shape CD4 differentiation towards a T-helper 1 (T_H1) phenotype. The environment in which activation occurs hence plays a crucial role in shaping T cell fate. APCs upregulate costimulatory molecules in an inflammatory context; for example conditioning chemotherapy-related tissue damage during HSCT results in release of damage-associated molecular patterns (DAMPs) such as ATP and uric acid. Signaling through TLRs and nucleotide-binding oligomerization domain–like receptors (NLRs) leads to inappropriate antigen presentation by a host of innate and adaptive cells. In the context of thymic injury, expression of tissue-restricted antigens and impaired negative selection, aberrant priming of autoreactive $CD4^+$ T cells and B cells can lead to chronic GVHD (221).

In addition to TCR signaling strength, surface molecules, and asymmetric division of metabolic regulators, tissue microenvironments are key to determining cytokine signaling and nutrient bioavailability. In particular mTOR integrates multiple environmental cues and cytokine inputs to promote protein synthesis and glycolysis via HIF-1α while the 5’ adenosine monophosphate-activated protein kinase (AMPK) complex inhibits mTOR and induces fatty acid oxidation. Other regulators of memory formation include the ratio of transcription factor pairs such as T-bet and Eomes or Blimp-1 and Bcl-6.

Large meta-analyses of gene expression profiles have not identified lineage-defining genes but instead have identified key networks of multiple regulators working in concert to promote particular memory or effector states (222, 223).

Finally, the relevance of chromatin accessibility is becoming increasingly considered in how we think about T cell memory. The remarkable variety of memory and effector phenotypes generated from clones with identical DNA sequences hints at the potential for therapeutic modification of 'stemness' and effector-associated genes via epigenomic alterations mediated by chromatin regulators such as histone methyltransferases (224). In place of the traditional terminology of 'fate', integration of multiple signal inputs of differing strengths is now thought to guide cells along a gradient towards or away from particular states (219). A key question in the field is how exactly these signals are integrated and the degree of influence that extrinsic tissue microenvironmental signals have over cell-intrinsic factors such as the epigenetic landscape.

1.10 CXCL12-CXCR4 in T cell biology

The role of CXCR4 and CXCL12 in T cell biology has been under-explored compared to what is known about its central role in haematopoietic stem cell and even B cell biology. CXCR4 is expressed constitutively at low levels on the majority of human $CD8^+$ T cells. $CD27^+CD28^+$ $CCR7^+CCR5^-$ naïve and central memory subsets express higher levels than effector cells (225). The greater protective immunity mediated by central memory $CD8^+$ T cells has

been suggested to be due in part to their greater migratory flexibility. Central memory $CD8^+$ T cells roll and adhere firmly to high endothelial venules in order to access lymph nodes, and this behaviour is dependent upon CXCL12, persisting even when CCR7 signaling is not present. In contrast naïve $CD8^+$ T cell lymph node migration is almost completely abolished by lack of CCR7 ligands (226), whilst both naïve and memory $CD4^+$ T cells arrest and adhere to integrin receptors following CXCL12 triggering (227).

The WNT family of secreted glycoproteins is known to be critical to the generation and long-term maintenance of memory $CD8^+$ T cells (228, 229). The WNT downstream effector transcription factor TCF7 (transcription factor 7 or T-cell factor 1, TCF-1) is highly expressed in naïve and early memory T cells and progressively lost with differentiation (230). Activation of the canonical WNT-β-catenin pathway promotes generation of T^{SCM} and T^{CM} (211) while Tcf-1-deficient $CD8^+$ T cells exhibit deficient T^{CM} formation, reduced CD122 and Bcl-2 expression, diminished responsiveness to IL-15 and impaired pathogen responses (231, 232). CXCL12-treated human T cells upregulate non-canonical Wnt pathway members such as Wnt5A, while down-regulating canonical family members such as Wnt3A. Wnt5A expression is required for CXCL12-induced migration in vivo and enhances CXCL12-CXCR4 signalling through protein kinase C activation (233), raising the prospect that altered CXCR4 expression may have multiple differing effects upon memory formation.

CXCR4 is also expressed upon naïve and memory $CD4^+$ cells and expression is upregulated by IL-4 in addition to the common gamma-chain cytokines. CXCL12 promotes $CD4^+$ T cell survival through the PI3K and MAPK pathways,

inactivated pro-apoptotic mediators and promoted responsiveness to TCR-mediated signals (234). Activated dendritic cells induce strong expression of CXCR4 on central memory $CD4^+$ T cells in a CD40/CD154 and CD134/CD134L dependent interaction (83). These interactions are exploited by HIV, allowing T cell tropic strains (X4) to interact with both CD4 and CXCR4 and adhere to the cell, inducing conformational changes in gp120/gp41 complex and membrane fusion (235). Indeed, mutations in SDF-1 confer much stronger protective effects against the onset of acquired immunodeficiency syndrome (AIDS) than CCR5 and CCR2 variants (236). CXCR4 expression is downregulated upon activation of T cells, in the opposing direction to expression of CD25.

The function of CXCR4 in T cells beyond migration is still unclear. CCR5 and CXCR4 are co-recruited into the immunological synapse and receptor cooperation is required for chemokine-mediated costimulation. This recruitment reduces T cell responsiveness to other chemoattractant sources and may serve the function of increasing the duration and stability of T cell-APC interactions and enhancing activation (237). Notably, CXCL12 and CCL3 (a CCR5 ligand) chemokine gradients are unable to dislodge effector T cells bound to ICAM-1 and MHC-peptide complexes, in contrast to ligands for chemokine receptors such as CCR7 and CXCR3 (238). Indeed, CXCL12/CXCR4 signaling has been shown to be necessary for immune synapse formation (239), phosphorylation of TCR-associated adaptor proteins (240) and thymic TCRβ-selection (241, 242).

CXCL12-CXCR4 signaling has been shown to activate multiple downstream pathways in T cells, including the extracellular signal-regulated kinases (ERK-

1 and ERK-2) (64, 243). The mechanism of pertussis toxin inhibition of $G\alpha_i$-protein-mediated chemotaxis involves activation of the TCR signalling network, which may reflect an evolutionary mechanism to prevent newly activated T cells from migrating away from antigen (244). This interaction is bidirectional, as close physical association of CXCR4 and the TCR results in phosphorylation of the intracellular TCR signaling apparatus and resultant AP-1 transcription and cytokine secretion (245). Recently, ligation of the TCR has been shown to trigger this association, trans-activating a CXCR4 signaling pathway resulting in stabilization of IL-2, IL-4 and IL-10 mRNA in naïve and memory $CD4^+$ T cells (246). It is therefore possible that altered T cell expression of CXCR4 will also modulate T-APC interactions with further effects upon differentiation and function.

1.11 T cell homing and the BM microenvironment

A wealth of evidence has suggested the importance of the BM in generating and maintaining memory T cells, as it does for other important components of the immune system (49, 68, 247, 248). Although T cell precursors migrate to the thymus for development of naïve T cells, the BM is a major reservoir for protective memory $CD8^+$ T cells, including central memory $CCR7^+$ $CD62L^+$ T cells (249, 250). These cells are preferentially recruited to the BM ahead of naïve and effector cells due to more efficient selectin and integrin activation and adherence to BM microvessels, and increased responsiveness to CXCL12 which is required for arrest prior to migration into the BM (3).

Naïve and memory T cells differ in their cytokine requirements. Naïve $CD8^+$ T cells require IL-7Rα signaling whilst memory T cells require IL-15 for basal proliferation although IL-7 is thought to have a role in mediating cell survival (251). In contrast, homeostatic proliferation of memory $CD4^+$ T cells demands a larger role for IL-7 in addition to TCR signaling and/or MHC class II molecules (252-254).

After antigen clearance, memory $CD4^+$ and $CD8^+$ T cells are maintained by slow turnover. The BM has been found by several groups to be the preferential site for homeostatic proliferation of memory CD8+ T cells. These cells express lower levels of the IL-7Rα (CD127) (255), suggesting that proliferation is IL-15 dependent as this cytokine is known to downregulate IL-7Rα expression in the BM (256) (257).

IL-15 also promotes the survival of naïve and memory phenotype $CD8^+$ T cells at much lower concentrations than those required for proliferation, via upregulation of survival signals such as B cell lymphoma 2 (BCL-2) and BCL-X_L (258), and inhibiting activation-induced cell death mediated by IL-2. Both IL-7 and IL-15 are known to increase the survival and frequency of early memory phenotype native T cells and autologous CAR-T cells, subsequently enhancing antitumour immunity via resistance to cell death (212, 259).

The precise nature of the signals received by T cells in the BM, and the phenotype of differing populations of memory T cells is controversial, with some groups demonstrating the existence of predominantly resting human memory T cells in G_0 phase of cell cycle (260), whilst other groups have found highly proliferative and activated populations (261). Analogous to

haematopoietic stem cells, the possiblity that separate niches exist to support both memory T cell proliferation and quiescence has been suggested (262). Shortly after acute infection, quiescent cells with lower CD25 and Ki-67 expression localise to the bone marrow (263). Quiescent human $CD8^+$ memory T cells can be mobilised from the BM using the CXCR4 antagonist Plerixafor (264).

This memory niche is thought to be organized by access to mesenchymal stromal cells producing IL-7 and IL-15 (265). In support of this concept, expression of IL-15 mRNA is much higher in CXCL12-abundant reticular cells than other BM cells and NK cells, which require IL-15 for development and maintenance, are mostly in contact with these reticular cells (68). High affinity IL-15 signalling is mediated by trans-presentation. This is the concept that IL-15 and the high affinity IL-15 receptor α subunit (IL-15Rα, CD215) are expressed by the same cellular source, and bind to memory T or NK cells expressing low affinity β and γ receptor subunits to form a high-affinity trimeric receptor (266-268). This mechanism is rarely found amongst cytokines and has the advantage of controlled cytokine delivery to appropriate cellular targets. CXCL12-abundant reticular cells therefore represent a putative niche that can trans-present IL-15 to memory T and NK cells (266, 269).

The BM is therefore a rich source of polyfunctional human memory T cells, important for defence against pathogens, and possibly also cancer, as suggested by studies highlighting the accumulation of memory $CD8^+$ T cells specific for tumour antigens such as WT-1 amongst others within the BM (270, 271). BM memory T cells display faster antigen responses compared to splenic counterparts (272). This is likely to be due to the fact that BM is an

important site for antigen-dependent priming of both $CD8^+$ and $CD4^+$ T cells, acting as a secondary lymphoid organ to mediate cognate interactions observed between clusters of dendritic cells and naïve T cells (273). Further studies have found that large clusters of $CD4^+$ memory cells (non-follicular helper type) engage in prolonged contact with B cells and antigen laden dendritic cells for multiple days post antigen boosting, resulting in pronounced numerical amplification. Peritumoural mature DCs in tertiary lymphoid structures are associated with extranodal activation, strong $CD8^+$ memory T cell infiltration and improved survival in human cancers (274, 275). In a similar way, the BM microenvironment could allow for the formation and maintenance of 'immune niches' as well as memory niches (276, 277).

The shared transcriptional program between long-term haematopoietic stem cells and memory T cells also raises the possibility of overlap between HSC and T cell niches within the BM (209). A great deal of attention has been paid to the constituents of the HSC niche, and as comparatively little known about the localisation and cells that comprise a $CD8^+$ T cell memory niche, a key question is whether similar cells can be identified in association with T cells in the BM. In order to ask relevant questions regarding the nature of a putative memory T cell BM niche, it is therefore important to review the key components of the HSC BM niche, with particular reference to CXCL12 production and signalling (Figure 1-4).

The precise nature of HSC stromal niches in the BM is a matter of complex ongoing investigation with multiple heterogeneous cell types expressing overlapping combinations of markers (278-282). The key stromal elements are perivascular cells, endothelial cells and osteoblasts, whilst sympathetic

nerves, non-myelinating Schwann cells, macrophages and osteoclasts also contribute to niche regulation. Sean Morrison's lab amongst others has shown that most HSCs reside close to vascular sinusoids within the bone marrow. The highest levels of CXCL12 are expressed by perivascular stromal cells (15,000-fold the level of unfractionated BM), then sequentially endothelial cells (120-fold), osteoblasts (13-fold) and some cells of haematopoietic origin (3-fold) (281). 90% of $CD45^{-}PDGFR\alpha^{+}$ $TER119^{-}$ mesenchymal cells express CXCL12, as compared to 70% of VE-$cadherin^{+}$ endothelial cells (281, 283) (Figure 1-4B).

Conditional deletion experiments have shown that HSCs are dependent upon a perivascular niche, composed of leptin receptor (LepR) or paired related homeobox 1 (Prx1)-expressing perivascular stromal cells, in addition to endothelial cells (284). The close spatial association of perivascular mesenchymal and endothelial cells may be therefore be important for cytokine-mediated cellular development and full niche functioning (285). Niche positioning also regulates cell cycle status. Quiescent HSCs associate with sparse $NG2^{+}$ Nestin-GFP^{bright} arterioles, as opposed to Nestin-GFP^{dim} sinusoids (286). Depletion of these periarteriolar cells induces HSCs into cell cycle and reduces long-term repopulation ability.

In contrast, early lymphoid progenitors (common lymphoid progenitors (CLP) and IL7Rα$^+$ lymphoid-primed multipotent progenitors (LMPP) occupy a spatially distinct niche dependent upon endosteal cells expressing the osteoblast-lineage type I collagen a1 promoter (Col2.3) (281, 282). Interestingly, committed B lineage progenitors have been shown to return not to this endosteal niche but a perivascular Prx-1$^+$ stromal niche. These Prx-1$^+$ cells overlap strongly with the above-mentioned CD45$^+$PDGFRα$^+$ TER119$^-$ mesenchymal cells and CXCL12-abundant reticular cells (52, 287, 288).

A niche for mature T cells, quiescent or otherwise, has not yet been identified. In vivo imaging has identified T_{reg} cells co-localising with HSCs on the endosteal surface of the BM post allogeneic transplant to provide immune sanctuary (289). T_{reg} cell trafficking to the BM is dependent upon CXCL12/CXCR4 (290), suggesting that the CXCR4/CXCL12 axis is one of the critical factors dictating the balance between tolerance and immunity in the BM as in other stromal and malignant niches (169, 291, 292). Given the frequency of bone marrow metastases in solid tumours, the bone marrow has been suggested as an immuno-suppressive microenvironment (293) and further detailed study of the interaction between inflammatory and regulatory populations within the niche is required. Murine bone marrow transplant (BMT) models have demonstrated that BM-derived CD8$^+$ T cells possess strong anti-tumour activity but induce lower rates of GVHD. The mechanism underlying this is still unclear but may relate to lower proportions of naïve or effector memory T cells (294, 295).

The prevailing view in the field is thus that tissue niches play a major role in determining cell fate in health and disease. Specifically, the bone marrow

niche is thought to be vital for the maintenance of normal haematopoietic stem cells. Leukaemic cells have been suggested to remodel this microenvironment to their own advantage, disrupting normal HSC niches and sequestering normal stem cells (296-298). Human acute myeloid leukaemia stem cells (LSCs) engraft in osteoblast-rich endosteal regions of murine BM, which may mediate apoptosis resistance via quiescence (299). In contrast, chronic myeloid leukaemia cells induce a reduction in BM CXCL12 expression, abnormal LSC localisation and suppression of normal HSC growth (300), suggesting that variations in disease-specific niches may require differing therapeutic approaches.

Recently, in vivo imaging of human T-cell acute lymphoblastic leukaemia (T-ALL) in a murine model from Cristina Lo Celso's lab has upended dogma by suggesting that T-ALL migrate dynamically across multiple BM regions rather than using a specific BM niche (301). Furthermore, chemotherapy resistant cells were highly migratory, suggesting that survival in this model may be stochastic rather than niche-dependent. Importantly, disease progression induces endosteal remodelling, resulting in depletion of mature osteoblasts while preserving perivascular mesenchymal stroma. This accords with other studies demonstrating osteoblast loss in acute myeloid leukaemia (AML) (302, 303) and dysfunctional osteoblast expansion in myeloproliferative neoplasia (304).

The Lo Celso study suggests the possibility that cellular therapies with similar migratory capacities to leukaemic T cells may be more effective than specific pharmacological interventions targeting the niche. Furthermore, disease-induced remodelling of the BM niche, in particular CXCL12-producing cells,

may alter the trafficking abilities of engineered therapeutic T cells in unexpected ways. For example, T-ALL express CXCR4 at higher levels than normal $CD4^+$ and $CD8^+$ T cells, hence over-expression of CXCR4 in therapeutic T cells may mimic and disrupt the ability of T-ALL to interact with multiple stromal components. Alternatively, loss of a physiological memory T cell niche may similarly affect the survival and function of therapeutic T cells. Defining the existence and nature of a cellular niche for therapeutic T cells is therefore of great importance in the era of adoptive immunotherapy.

Despite the important contributions of the studies above, no studies have yet shown that directly modulating T cell trafficking to the bone marrow alters phenotype. Higher CXCR4 expression on pathogenic T cells in immune-mediated aplastic anaemia facilitates BM entry, suggesting a viable mechanism for improving T cell homing to immune niches within the BM (291). Of relevance to this concept, genetic deletion of CXCR4 in mature $CD8^+$ T cells does not alter antigen-dependent expansion but markedly reduces homeostatic self-renewal and memory T cell frequency, particularly in the BM (305).

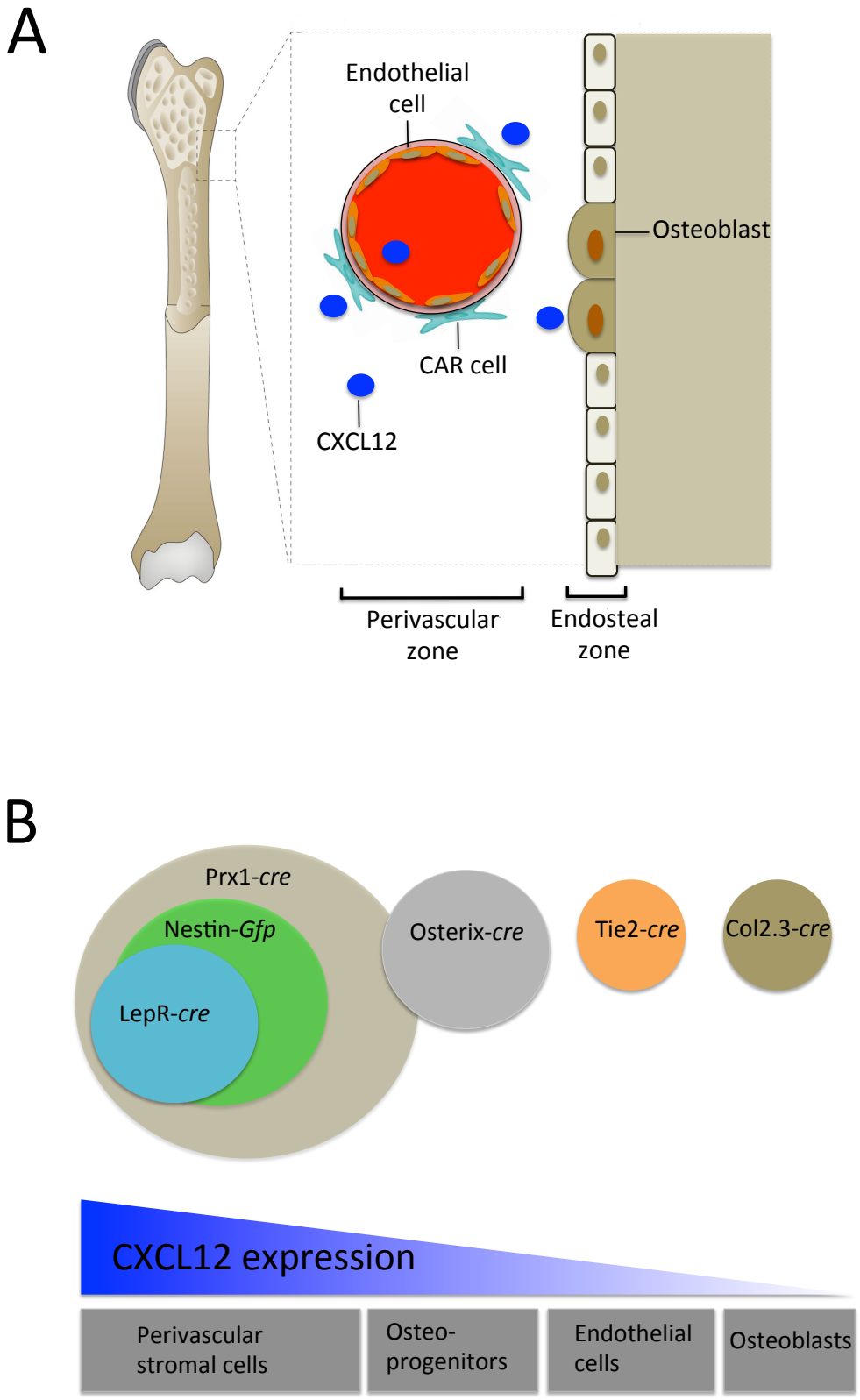


Figure 1-4 The stromal BM microenvironment.
(A) The bone marrow niche comprises many different cell types

residing within the marrow cavity. The interface between bone and bone marrow, known as the endosteum, contains bone-forming osteoblasts. Many haematopoietic cells reside close to blood vessels, particularly sinusoids, which allow cell migration from the circulation, and oxygenated arterioles. Vascular structures are covered on the lumenal side by endothelial cells, and ensheathed by perivascular stromal cells, including CXCL12-abundant reticular (CAR) cells. (B) Schematic indicating the degree of CXCL12 production in various stromal populations. Perivascular stromal cells targeted by Cre-mediated recombination of identifying transcription factors (Prx1 and LepR) overlap with Nestin-*Gfp*$^{+}$ cells in producing the largest amounts of CXCL12, and progressively lower amounts are produced by osteo-progenitors (marked by Osterix-*cre*), endothelial cells (Tie2-*cre*) and osteoblasts (Col2.3-*cre*).

1.12 Immunotherapy and its challenges

1.12.1 The range of immunotherapeutic agents

The possibility of redirecting the immune system to treat cancer has been well described for some time now. In 1891 William's Coley's pioneering streptococcal injections into sarcomas to stimulate an immune cycle were published as case series' and not taken up widely by the field. The dangerous side effects, including systemic fever, were cited as a major disadvantage. Over one hundred years later, bone marrow transplantation is well established as a powerful treatment for haematological malignancies but there is now increasing interest in manipulating the immune system to evoke specific immunogenicity against neoplastic cells. Active agents currently being explored include T and DC growth factors and agonists, antagonists of immunosuppressive cytokines, immune checkpoint inhibitors (CPI), vaccines and cellular therapies (including CAR-T cells, TCR-modified T cells, tumour-infiltrating lymphocytes (TILs), NK and gamma delta T cells).

Immune growth factors and agonists

The original 'T-cell growth factor', IL-2, was discovered in 1976 and greatly facilitated experimental exploration of the T cell properties. Although approved for use in metastatic renal cell carcinoma and melanoma, high expression of IL-2Rα (CD25) on T_{reg} cells potentiates a dual immune effector and regulatory function, raising concerns over excessive differentiation and limited anti-tumour utility (306). Memory T cells generated without IL-2 predominantly express a central memory phenotype and retain CD62L expression and IL-2 secretory capacity. In contrast to the usual paradigm of CD4+ 'helper' T cells providing the majority of IL-2, autocrine $CD8^+$ T cell IL-2 secretion is thought to permit memory expansion (307, 308). This concept suggests complementary and opposing roles in immunity for the other common gamma chain cytokines, particularly IL-15.

Human IL-15 is encoded close by IL-2 on the short arm of chromosome 4, and binds to the same β (CD122) and γ receptor subunits (CD132), yet shares little homology with IL-2 at the protein level and initiates a distinct gene expression program culminating in maintenance of memory $CD8^+$ T and NK cell homeostasis with limited differentiation (309). The mechanism of this distinctive effect is not fully understood but may be mediated by the in vivo requirement for trans-presentation (310). This model suggests that IL-15 acquires some of the property of a cell surface molecule, requiring close physical proximity for activity and potentially mediating more potent signaling in a niche environment. This makes immuno-regulatory sense, preventing formation of an intrinsic amplification loop through memory $CD8^+$ T expression of IL-15Rα. Trans-presented IL-15 was initially suggested to be primarily

dendritic cell and monocyte-derived; however recent reporter mice experiments have demonstrated non-haematopoietic stromal niches to be the major sources, particularly CXCL12-abundant reticular cells in the BM (311).

IL-15 enhances antitumour activity of $CD8^+$ T cells in poorly immunogenic tumours (312) and reduced levels of IL-15 are associated with malignancy relapse post allogeneic bone marrow transplant (313). IL-15 is the number one target in a list of twelve immunotherapy drugs formulated by the US National Cancer Institute (NCI) as having the greatest potential to cure cancer in novel combination therapies (314). In inflammatory settings, IL-15 permits more rapid division of memory cells after antigen encounter and enhances protective capacity (315). Studies in IL-15 deficient mice show similar deficits in $CD8^+$ memory T cells to mice lacking CXCR4 (316).

IL-15 also induces expression of several costimulatory TNF receptor family proteins such as glucocorticoid induced TNFR-related protein (GITR) and 4-1BB (CD137) on BM memory $CD8^+$ T cells in contrast to other secondary lymphoid tissues such as spleen and LN (317, 318). 4-1BB is required on T cells for maximal recall responses and more accurately identifies tumour-reactive TILs than PD-1 (319). 4-1BB is more highly expressed on tumour-reactive and BM memory $CD8^+$ T cells compared to spleen and LN, in an antigen-independent mechanism dependent upon IL-15 (318, 319), while 4-1BBL expression on VCAM-1^+ BM stromal cells provides survival signals downregulating pro-apoptotic Bim signaling in BM $CD8^+$ T memory cells (320).

Engineering of costimulatory domains is an active area of study for researchers aiming to shape the effector/memory balance and functional

capacity of therapeutic T cells. Inclusion of a 4-1BB intracellular domain in chimeric antigen receptor T cells (CAR-T cells) enhances in vitro persistence by increased fatty acid oxidation, forms superior quality immune synapses and enhances anti-tumour efficacy (321-323) while CAR-T cells incorporating CD28 domains demonstrated enhanced aerobic glycolysis characteristic of effector T cells (324). Intratumoral $CD8^+$ T cell apoptosis has been shown to be a major component underlying T cell dysfunction and preventing effective antitumour immunity in mouse models (325), while a concern regarding tonic CAR-T cell CD3-ζ phosphorylation is the early induction of exhaustion, limiting anti-leukaemia efficacy (326). Mechanisms for enhancing T cell persistence in these settings are hence much sought after. A 4-1BB agonist antibody combined with checkpoint inhibition increases accumulation of TILs through enhanced survival rather than proliferation, resulting in synergistic tumour control in murine models, and suggesting the potential for further exploration of this idea (325).

A cautionary note is provided by the observation that IL-15-dependent NK cells can develop ageing-related dysfunction and impaired cytotoxicity due to defective maturational cues from BM stromal niches (327). Research targeting BM stromal niches as a source of cells for immune therapy must consider the effects of an ageing niche and proinflammatory cytokines. IL-6 and IL-15 are known to be increased in the ageing human BM, as are T^{EM} and a putative highly activated $CD28^-CD57^-$ $CD8^+$ T cell population, and this may have relevance for the use of adoptive immunotherapies in older people and damaged stromal niches (328).

IL-7 is similarly known to promote $CD8^+$ T cell survival in both the naïve and memory setting. It signals through a heterodimeric receptor composed of IL-7Rα (CD127) and CD132 but is thought to be more important for $CD4^+$ T cell development and homeostasis. CD127 expression has been suggested as a marker of $CD8^+$ T cells fated for long-term memory development (329). Limiting secretion from non-haematopoietic stromal niches in the thymus and bone marrow is enhanced during periods of lymphopenia and induces expression of BCL-2 family members (330). Recombinant IL-7 has shown pre-clinical promise in amplifying polyfunctional $CD4^+$ antitumour effects and immune reconstitution, but may also potentiate B-cell malignancies, and aggravate GVHD in the post-allogeneic transplantation setting, limiting its utility (331-334).

IL-21 is another common gamma chain cytokine of clinical interest in promoting a less differentiated $CD27^{high}CD28^{high}$ $CD45RO^+$ $CD8^+$ T cell phenotype. Produced by activated $CD4^+$ T cells, it is non-essential for lymphoid development but synergises with IL-7 and IL-15 to promote naïve and memory $CD8^+$ T cell proliferation and IFN-γ expression (335-337). Finally, IL-12 has been suggested as a potent anti-tumour cytokine, primarily secreted by activated APCs, shaping a T_H1-biased innate and adaptive immune response. Despite this mooted potency, clinical trials have been limited by toxicities, development of adaptive negative feedback to IFN-γ generation and a myeloid-coordinated immunosuppressive milieu (338, 339). Combination approaches are under investigation, such as T cells redirected for universal cytokine-mediated killing (TRUCKs), which aim to deposit IL-12 in the tumour and unleash an immuno-stimulatory cycle (340).

Checkpoint inhibition

As more became known regarding the role of immune evasion in cancer, a handful of researchers identified proteins on T cells that appeared to inhibit their action. CTL activation gene number 4 (CTLA-4) was initially identified in activated T cells but despite sequence homology with the pivotal costimulator CD28, it was found to have an inhibitory role, binding with significantly greater binding affinity to the B7 ligands than CD28 (341, 342). Researchers interested in apoptosis first identified programmed cell death protein 1 (PD-1) and further collaborations identified its ligands as homologous to the B7 ligands (343, 344). The finding that deletion of either of these molecules results in autoimmunity led to the key conceptual breakthrough - the immune system as a finely tuned network of opposing forces, ripe for exploitation. This equilibrium, termed the 'cancer-immune set point', is defined as the balance between factors acting to enhance and subdue antitumour immunity (345). The fact that T cell stimulation in the absence of a vaccine can induce disease regression further suggests a requirement for therapies able to potentiate latent tumour-specific potential within many patients. The discovery that the newly discovered PD-1 ligand (B7-H1) is not expressed in normal tissues but upon a range of human cancers, and that expression is upregulated upon exposure to IFN-γ, led to the conclusion that cancer cells have hijacked the utility of co-inhibitory molecules to evade immune attack (346). Previous attempts to harness immune potency by vaccination had elicited weak and ineffective responses, but the fatal lymphoproliferative disease seen in *Ctla4* knockout mice, suggested the potency of CTLA-4 compared to CD28 as a means of dramatically enhancing these weak responses. James Allison and

others were subsequently able to prove that this approach could enable checkpoint inhibition as an entirely novel method of cancer treatment (347).

The differences between patterns of checkpoint molecule expression on immune subsets have allowed for experimental dissection of their mechanisms, and potential utility in different scenarios. CTLA4 is expressed only upon activation, predominantly by T_{reg} cells. Inhibition is associated with a high degree of autoimmune side effects, suggesting that blockade or depletion of T_{reg} cells may be the major mode of action (348). In contrast, PD-1 is expressed on activated T and B cells but is sustained in a subset of antigen-specific T cells rendering them inactive in chronic viral infection and cancer (349, 350). IFN-γ produced by cytotoxic T lymphocytes is a key pathway targeted for immune evasion by upregulation of the key checkpoint ligands PD-L1 and PD-L2 on malignant and accessory cells (351). The hallmarks of exhausted PD-1^{HI} cells are co-expression of a host of inhibitory receptors such as Tim-3 (T cell immunoglobulin and mucin domain-containing protein 3), Lag-3 (lymphocyte activation gene 3) and TIGIT (T cell immunoglobulin and ITIM domain) amongst others. They are poorly responsive to common gamma-chain cytokines such as IL-2, IL-7 and IL-15, have impaired homeostatic proliferation and a reduced capacity to produce IFN-γ, IL-2 and TNF-α upon re-stimulation (352).

Melanomas as the prototypical immune-sensitive tumour are highly mutated (0.5 to >100 mutations per megabase) and have been shown to be highly sensitive to immune checkpoint blockade by antibodies to CTLA-4 and PD-1 (353). Patients with a higher mutational burden were more likely to experience durable clinical responses, hinting that the genetic diversity of cancer may be

an Achilles heel in the context of immunotherapy. Responses to PD-1 blockade in melanoma patients have been shown to correlate with increases in the number of $CD4^+$ central memory T cells (354). Importantly, patients responding to CPI show a survival plot plateau at 2 years, suggesting that long-term immunosurveillance can produce functional cures (355).

In efforts to analyse the mechanisms underlying some of the extraordinary responses to PD-1 blockade in advanced cancer, some groups have defined a $CXCR5^+CD8^+$ T cell subset that preferentially proliferates after antibody treatment in chronic viral infection and express higher levels of co-stimulatory receptors such as OX40 and 4-1BB. They are enriched for genes associated with mitochondrial fatty acid oxidation and exhibit a transcriptional profile similar to memory precursor cells, including markedly enhanced expression of CXCR4 (356, 357). In contrast to the primarily metabolic effects of PD-1 blockade, profiling of TILs post CTLA-4 blockade show changes in cell cycle and effector memory pathways (358). Further studies in viral infection have highlighted heterogeneous subsets of $PD\text{-}1^+$ T cells with differing roles. 'Progenitor-like' $T\text{-}bet^{HI}$ $PD\text{-}1^{INT}$ $CD8^+$ T cells are more susceptible to the proliferative effects of CPI, but can develop into the terminally differentiated $Eomes^{HI}$ $PD\text{-}1^{HI}$ subset, which localise to peripheral tissues and lack the capacity for restoration of T cell functions, possibly due to lack of CD28 (359, 360). The ratio of the magnitude of T cell response to tumour size has been directly correlated with anti-PD-1 response in metastatic melanoma, highlighting the importance of the size of the $PD\text{-}1^+CXCR5^+$ population (361).

Although PD-1/PD-L1 inhibitors have mediated powerful responses in tumours such as melanomas and non small cell lung cancer, these tumours

bear a median of 200 nonsynonymous mutations per tumour (362). The response to CPI correlates with the tumour mutation burden and PD-L1 expression (363, 364). Other cancers such as acute myeloid leukaemia express lower numbers of mutations. These cancers are expected to be less intrinsically immunogenic, and may also deploy additional immune evasion strategies. Furthermore, recognition of mutant antigens is inefficient; only a fraction of peptides derived from potential neoantigens are in fact immunogenic. Rational strategies are also required to overcome the key issues of (a) defective homing to the tumour site and (b) an immunosuppressive microenvironment. Durable complete responses (CRs) to CPI are limited to a small subgroup (<20%) in most studies, whilst off-target treatment-related adverse events are extraordinarily common, and frequently severe (365). As with the notable examples of curative chemotherapeutic regimes, combination therapy is likely to be required to increase cure rates, in addition to strategies targeting multiple tumour-associated antigens. Recent novel work has hinted at the importance of enhancing T cell homing; alterations of microbiome can enhance efficacy of PD-1 blockade in an IL-12 dependent manner by inducing recruitment of $CCR9^{+}CXCR3^{+}CD4^{+}$ T cells into the tumour (366, 367).

Cellular therapies and vaccines

Preliminary studies have suggested the effectiveness of combination PD-1 pathway blockade and adoptive T cell therapies in increasing T cell persistence, activation and chemokine homing capacities (368, 369).

Generation of T cells specific for mutation-associated peptides and personalized vaccines has also been shown to be feasible. Use of vaccination to enhance engineered T cell homing to solid tumour sites is therefore an attractive approach to overcome defective localisation (370). Further combinatorial approaches using novel nanomaterial-based delivery mechanisms, dendritic-cell based vaccines, oncolytic viruses or adoptive therapy with novel combinatorial CAR-T cells or TCR-transduced T cells are likely to be incrementally more successful (371, 372). Current CAR-T protocols have yielded historically high responses rates in the relapsed/refractory setting of $CD19^+$ haematological malignancies but toxicity is an issue and relapses are frequent in the adult setting, only some of which relate to antigen-loss variants (373, 374). There is therefore an important therapeutic need for cellular therapies suitable for older patients and mediating long-term disease control.

A recent example used established granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing cellular cancer vaccines formulated with cyclic dinucleotides to induce type 1 interferon responses and hence DC and $CD8^+$ T cell activation. This approach uses pathogen-associated molecular patterns (PAMPs) to elicit activation of the innate immune system via the stimulator of interferon gene (STING) signaling pathway. This can in turn be harnessed to effective adaptive antitumour immunity by combination with PD-1 blockade (375). Other novel tactics include using anti-tumour TCR-engineered cells with a second TCR recognizing a bacterial antigen to enhance tumour homing and reverse microenvironment immunosuppression, consistent with the known effects of pathogen-based immunotherapies in

altering the phenotype of MDSCs and inducing IL-12 production (376-378). Steve Rosenberg at the National Cancer Institute, a pioneer in the field of genetically modified lymphocytes to treat human cancer, has achieved durable remission in hard-to-treat large breast and liver tumours using dual-specific CAR-T cells and tumour antigen vaccination incorporating vaccinia virus. In this setting considerable T cell infiltration was achieved in a CXCR3-dependent pattern (379).

Combination regimes and synthetic niches

It may be also possible to combine therapies enhancing a particular cell fate with synergistic molecular agents. Demethylating agents (DNMTis) such as azacitidine and decitabine are active agents in treatment of myeloid leukaemias and are known to increase expression of multiple endogenous retroviruses (ERVs) and upregulate tumour-associated antigens and co-stimulatory ligands. This leads to induction of a double-stranded RNA (dsRNA) sensing pathway and enhanced T cell infiltration (380-383). There is potential for synergistic use of DNMTis in combination with memory T cells specific for leukaemia-associated antigens such as WT-1, MAGE-A and PRAME. This allows for targeting of resistant or quiescent tumour stem cell populations responsible for relapse. Encouraging historically high complete responses to azacitidine and PD-1 blockade combination therapy in relapsed AML are associated with progressive increases in BM $CD8^+$ T cell infiltration, suggesting the importance of enhanced lymphocyte homing (384).

The use of radiation or poly ADP ribose polymerase (PARP) inhibitors to prevent repair of single-strand DNA breaks, can synergize with CPI, linking chronic DNA damage with formation of a proinflammatory immune environment involving enhanced chemokine production (385, 386).

Other strategies to mobilise patient T cells in site-specific approaches are promising. A direct pre-clinical approach involves injecting a TLR9 ligand directly into tumour to induce expression of the TNFR superfamily costimulator OX40 on intratumoral CD4 T cells. Combination therapy with an anti-OX40 antibody resulted in cure of the majority of mice in a variety of tumour models, in contrast to combination with anti-PD1 antibodies. Interestingly, the mechanism here seems to involve OX40 stimulation directly impairing T_{reg} immune suppression, resulting in myeloid cell infiltration, and $CD8^+$ T and NK cell activation (387). Further approaches in this vein to break tumour-specific tolerance are likely to be highly effective because they target the microenvironment without inducing systemic toxicity.

Synthetic immune niches represent another promising concept to reprogram an antitumour response. Reminiscent of tertiary lymphoid structures, they consist of biomaterial-based scaffolds designed to mimic lymph nodes. The porous structure acts as a finely tuned delivery vehicle for T memory and other immune effector cells, loaded with antigen-presenting cells and immunostimulatory ligands. This provides the advantage of retaining cells in a tumour-specific location while providing them with activation cues such as IL-15 superagonists, cytokines and CpG oligonucleotides, and has been shown to be more effective than directly injected ex vivo stimulated T cells in the presence of an immunosuppressive microenvironment (388-390). The

scaffolds can be tailored to the precise type of tumour targeted, in terms of pore structure and biomolecule integration. For example, chemokine receptor-expressing T cells could be induced to localise towards a scaffold expressing the reciprocal chemokine for optimal in vivo activation at the tumour location (391). Experimental models have demonstrated important roles for $CD8^+$ DCs and plasmacytoid DCs (pDCs) in association with heightened local concentrations of IL-12 and GCSF in successful melanoma vaccines (392, 393). These therapies can be combined with agents that directly counter immuno-suppressive microenvironments such as immune checkpoint and TGF-β inhibitors (394-396). Considerable hurdles need to be overcome in terms of understanding these complex molecular and physical interactions, selecting rational combinations from a bewildering choice, limiting chronic inflammation and toxic degradation, and ensuring the safety of complex cell and biomolecule-based systems.

Haematopoietic stem cell transplantation

Whilst awaiting the development of precision immunotherapies effective against molecularly targeted populations, the optimal treatment for many poor-risk haematological malignancies remains the original immunotherapy – allogeneic haematopoietic stem cell transplant. This is still the most powerful and durable anti-leukaemic treatment yet developed, predicated as it is upon adaptive immune recognition of minor histocompatibility antigens expressed by host leukaemic cells. In this regard, strategies to enhance the survival and persistence of anti-viral T cells in the setting of delayed immune reconstitution

and life-threatening infection, or to enhance the graft versus leukaemia (GVL) effect mediated by donor lymphocyte infusions without triggering GVHD are much needed. Precise mechanistic separation of GVHD from GVL has not yet been achieved in the clinic. Use of adoptive cellular therapy in conjunction with inducible suicide genes could mitigate this risk, reducing the element of risk accompanying every transplant currently performed. One concern regarding use of potent $CD62L^+$ early memory phenotypes is the possibility of increased incidence and severity of GVHD, hence experimental demonstration of utility will also need to provide of safety (397). Interestingly, the use of CD19 CAR-T cells of defined $CD8^+$ T^{CM} and $CD4^+$ T cell composition post-allograft was associated with very low levels of GVHD in clinical studies (398, 399). This is in stark contrast to the rates of severe treatment-refractory GVHD post allograft and immune-related adverse events generally seen with CPI such as the PD-1 antibody nivolumab, and suggests an advantage for cell-specific therapy in an immune-inflamed environment (400).

Targeting somatic neoantigens

A number of lines of evidence support the proposition that targeting somatic neoantigens (the products of unique patient-specific tumour mutations) will eventually become a definitive and potent form of cancer immunotherapy (401). Tumours with high somatic mutation burdens are particularly responsive to immunotherapy. Driver mutations are by definition required to enable tumour growth hence targeting is expected to cause tumour death or

loss of fitness. Secondly, the exclusivity of neoantigens renders toxicity to normal tissues highly unlikely. Thirdly, T cells expressing TCRs specific for new tumour neoantigens are unlikely to have been deleted by central tolerance during thymic education and may well be present in patient's blood. TILs extracted from patients with melanomas induce durable CRs with minimal autoimmune toxicities, in contrast to CPI and TCR gene-engineered T cells targeting pigment antigens (162, 402). CD19 is in many respects an exception in that elimination of expression in normal tissues by B cell depletion can be safely managed. Targeting differentiation, cancer germline and over-expressed antigens expressed on normal tissue such as carcinoembryonic antigen (CEA) can mediate severe toxicity and are likely to be ineffective, in part due to high-avidity TCR-bearing cells undergoing thymic deletion (403, 404).

Somatic patient-specific neoantigens can now be readily identified. The rapid availability of whole exome and transcriptome sequencing can be used to analyse circulating tumour cells (CTCs) or biopsy specimens for founder mutations likely to be expressed in all tumour cells (405-407). Mutation-encoding minigenes can then be introduced into autologous antigen-presenting cells and cultured with PD-1^{+} patient T cells to identify tumour-reactive T cells. These TCRs can then be sequenced and used to modify expanded patient T cells for re-infusion. This method has been used to identify tumour-reactive T cells even in patients with low mutation burden cancers (406, 408). Furthermore T cells from one patient were found to target the $KRAS^{G12D}$ hotspot common to many human cancers, suggesting potential widespread utility of this technique in targeting clinically important 'trunk'

mutations (409). As yet, these T cells cannot mediate high remission rates, again highlighting the need for improved methods to enhance potency and persistence of the transferred cells. Key determinants of immunogenicity include the binding affinity between mutated peptides and MHC (410) and downregulation of antigen processing and presenting machinery (411, 412). Overcoming these challenges may require use of combination therapies targeting intratumoural heterogeneity and the genetic instability that is the hallmark of cancer.

1.12.2 The cancer immune set point

A successful anti-tumour cellular therapy must surmount several hurdles. Immune cells must be able to traffic to the tumour site, overcome local immune evasion strategies to infiltrate and recognise tumour antigens as foreign, expand and successfully destroy large tumour burdens, all whilst minimising off-target systemic tissue damage. As a means of understanding how to rise to these challenges, histological data investigating the low frequency of responses to anti-PD-1 pathway antibodies has been interrogated to yield three conceptual types of immune profile (413, 414).

The immune-inflamed profile comprises frequent T cells within the tumour parenchyma, associated with checkpoint ligand expression on tumour cells and suppressive populations. Responses to CPI are most often associated with this profile, suggesting a pre-existing immune response requiring potentiation. The rate-limiting step in this profile is therefore the differentiation and exhaustion phenotype of the T cell (415).

In contrast, the immune-excluded phenotype constitutes abundant T cells in the tumour stroma, suggesting the evasive strategy has limited their penetrative ability. The rate-limiting step here constitutes T cell homing to the tumour parenchyma. Finally, the immune desert phenotype contains few T cells, presumably due to non-immunogenic tumours carrying few neoantigens, and subsequent lack of a native anti-tumour T cell response as the rate-limiting step (345). These phenotypes represent a useful simplification of the vast diversity of immune evasion strategies, to aid in design of differing therapeutic strategies.

In the first profile, exhausted T cells acquire transcriptional and epigenetic modifications distinct from memory cells, rendering the majority insensitive to anti-PD-1 blockade alone. Successful antitumour cells may therefore require a persistent early memory phenotype despite repeated antigenic stimulation. The response to homeostatic cytokines (IL-7) is augmented by PD-1 blockade, suggesting a rationale for combination therapies focusing on these pathways. This could be performed in conjunction with genetic modifications aiming to limit exhaustion and re-activate important transcription factors, perhaps through modification of PDCD1 gene enhancer regions (416, 417). The role of the microbiome in enhancing systemic immunity will also be highly relevant (366, 367).

In contrast, the non-inflamed microenvironments of immune-excluded and immune desert profiles require the generation of a functional anti-tumour response able to successfully expand and target the tumour. T^{CXCR4} have demonstrated enhanced and functional capacities fitting these specifications. In addition, their homing capacities have been engineered to be particularly

relevant to the requirements of haematological malignancies. One could envision the infusion of early memory T cells equipped with TCRs recognizing disease and pathogen-specific antigens and chemokine receptors specialized for particular tumour profiles, in conjunction with intratumoral pathogen-based vaccination to enhance inflammatory signalling (376). Increased leucocyte chemokine levels have been noted in association with complete responses to CTLA-4 blockade for relapsed extramedullary acute myeloid leukaemia post allogeneic stem cell transplant (418). This suggests a further pathway for exploiting low mutational burden tumours, and also hints at the importance of exploiting lymphocyte trafficking in specific high-risk situations.

Further useful combination strategies to promote antitumour responses could involve targeting angiogenesis and epigenetic repression of chemokine expression. Expression of vascular endothelial growth factor-C (VEGF-C) in melanoma potentiates cancer immunotherapy via enhanced CCL21 and T cell tumour infiltration, hence strategies enhancing angiogenesis may be effective in inducing tumour-associated inflammation (419). Tumour-intrinsic β-catenin signalling and epigenetic silencing of genes encoding T_H1-type chemokines such as CXCL9 and CXCL10 represses T cell infiltration and impairs patient survival (420-422). Pharmacological epigenetic modulation such as β-catenin suppression and enhancer of zeste homologue 2 (EZH2) and DNMT inhibitors (421, 423-425) could be combined with adoptive cellular therapy incorporating memory T cells to render the tumour immunologically ‘hot’ and promote potent T cell recruitment.

Needless to say, the vast range of cancers represent a continuum of multiple immune evasive strategies and the success of therapies may depend

stochastically on the balance of immune suppressive and inflammatory phenotypes in the individual patient. The cancer-immune set point is therefore a useful theoretical concept to define the threshold to be reached for successful anti-cancer treatment in groups of patients with specific molecular profiles, to guide the design of rational combination therapies (345).

1.12.3 Challenges to the delivery of successful immunotherapies

The complexity of genetic analysis and manufacturing cellular therapies to GMP standards represents a major challenge to existing clinical infrastructure that has traditionally deterred pharmaceutical investment. The step-change in the clinical environment following on from the dramatic success of CD19 CAR-T cell and CPI therapies has completely altered this dynamic. Novel mechanisms for exploiting the potential of the immune system are rapidly being investigated. There are however a number of different technical, financial and regulatory hurdles before this success can be replicated in a wider range of cancers.

The first issue is the lack of truly tumour-specific antigens suitable for targeting by the single-chain variable fragment (scFv) of CAR-T therapies. The majority of antigens currently under investigation such as mesothelin are also expressed to varying levels by normal tissues and there is potential for considerable on-target off-tumour toxicity (426-428). The solutions to this could involve use of safety switches to delete or regulate CAR-T activation. Suicide genes such as herpes simplex virus–thymidine kinase (HSV-TK) or inducible caspase-9 (iCasp9) have been shown to be effective, but can also

compromise the anti-tumour response (429, 430). A more subtle approach under investigation is to engineer CAR-T cells to require two separate tumour associated antigens for full activation (logic-gated CARs), inhibitory CARs (iCARs) or CARs in two parts requiring infusion of a small-molecule dimerising agent for precision assembly (ON-switch CARs) (431-433).

A second solution is to find antigens uniquely expressed by the tumour in question, such as virally encoded genes (human papillomavirus) and driver gene mutations (*KRAS*, *NRAS*, etc) (434, 435). Heterogeneous tumour expression and selection for antigen-negative cells could compromise responses however, as would the requirement for compatible HLA haplotypes (436, 437). In this respect, an important development would be a clinical pipeline for targeting patient-specific neoantigens using autologous T cell therapy (401, 438). This pathway has been highly refined and offers the advantage of direct testing of $CD8^+$ and $CD4^+$ T cell reactivity to multiple neoantigens. Firstly, next-generation sequencing of tumour versus germline tissue identifies all non-synonymous mutations and constructs are synthesized encoding the mutant gene plus flanking codons. Autologous dendritic cells can then be transfected with construct RNA, or pulsed with mutant peptide, allowing for processing and presentation. We can then measure T cell reactivity to these cells in tandem using cytokine or activation molecule-based readouts (439).

This workflow has been demonstrated with some success in treating patients with metastatic cancer, even in cases lacking high mutation burden or evidence of DNA mismatch repair (406, 409). It offers the advantage of targeting a number of mutations simultaneously, combating Darwinian

selection from heterogeneous clones (440). This approach benefits from avoiding limitations associated with current algorithms, such that of hundreds of predicted neoantigens, only a handful generate proven T cell responses (358, 370, 406, 441). Improvements in understanding of proteasomal processing and MHC-binding stability will also improve the accuracy of predictive mathematical modeling (442).

Any strategy involving targeting of neo-epitopes must contend with the Red Queen effect: a successful strategy will become a victim of its own success due to selection for resistance (443). This risk is neatly highlighted by a recent case study using combined immunogenomic approaches to characterise immune infiltration into spatially distinct ovarian cancer metastases in one patient whilst off treatment (444). In a fascinating example of immune evasion at work, regressing metastases were infiltrated by oligoclonal expansions of T cells whilst progressing lesions displayed minimal infiltrates. Interestingly, site of later metastases had the highest neoantigen loads but no predicted neoantigens were shared between differentially responsive sites. Importantly, when the evolutionary relationship between tumour samples was reconstructed, truncal somatic mutations displayed lower immunogenicity scores than branched subclonal mutations. This, coupled with the lack of immune infiltrate in the primary tumour, suggests that mutations shared by every tumour cell are most likely to be eliminated, highlighting immunogenomic heterogeneity as a barrier to successful immunotherapy.

Destruction of the tumour cell is known to release further tumour-specific antigens to increase the depth of any adaptive immune response via cytokine and chemokine amplification. A generalizable approach will likely require

synchronous targeting of multiple neoantigens associated with different HLA molecules to overcome heterogeneity. Single melanoma antigen (MAGE) vaccination or Epstein-Barr virus (EBV)-targeted T cells can mediate T-cell destruction of MAGE/EBV antigen-negative tumour cells and contribute to remissions (445, 446). This epitope spreading concept views immunotherapy as kick-starting a cancer-immunity cycle and providing momentum to overcome micro-environmental restraint (447). In contrast traditional chemotherapy has often served as an accelerant, selecting for more aggressive disease variants, without the ability to adapt (448, 449). The adaptive and long-term antitumour immunity generated by immunotherapy make this approach more attractive as a strategy.

Serial monitoring of peripheral blood, tumour and immune response over time would potentially allow for detection of immune escape, such as alternative splicing of CAR-T targets in B-ALL (450). This would then allow for use of combination therapies to mitigate antigen–loss variants, or approaches aimed at altering tumour evolution through synthetic lethality, metronomic therapy and downstream targeting. Ultimately, the stochastic nature of tumour evolution can potentially be viewed as an Achilles heel, in that peripheral blood monitoring of tumour evolution and immune counter-response can be used to design effective extinction therapies (451), using two complementary therapies such that resistance to one entails susceptibility to the other in an evolutionary ‘double-bind’ (452).

Response rates to TIL and TCR-modified therapies are currently limited by a number of factors including lack of potency and persistence, possibly because the expansion process enriches for terminally differentiated cells. The ability

to transfer cells of high functional potency able to expand rapidly and persist long-term is potentially important to ensure durable responses (192, 212). Other alternatives such as reprogramming induced pluripotent stem cells into T cells are still in the early stages of development, limited by ongoing investigation into immune rejection, reprogramming efficiency and risks of malignant transformation (453-455).

The complex regulatory process involved in manufacture of GMP-grade viral vector has also introduced delays into the transfer of autologous neoantigens-targeted therapies to the clinic. A streamlined approach to the high financial and temporal costs of replication-competent retrovirus testing has been proposed (456). Non-viral alternatives such as the *Sleeping Beauty* transposon/transposase method (457) and genome editing strategies promise to make this process cheaper, faster and safer (458).

1.12.4 Safety concerns and toxicities

Based upon the data presented above, modifying therapeutic $CD8^+$ T cells to enhance homing to niches mediating improved persistence and function would seem an important and plausible strategy to improve upon current outcomes. Use of pre-conditioning regimes to increase accessibility of homeostatic cytokines, deplete suppressor populations and enhance antigen expression are important components of successful adoptive therapy (459). One strategy to mediate successful therapy has been suggested by analysis of CAR-T cells trials targeting multiply relapsed/refractory patients with bulky advanced-stage lymphoma. In this setting, the proportions of infused

autologous central memory CAR-T cells were directly associated with peak blood CAR-T levels, which in turn were associated with high serum IL-15 levels and clinical remissions. This confirms that T cells from older patients previously exposed to multiple courses of chemotherapy can be successfully re-directed to target tumours by access to the correct molecular signals however neurologic toxicity in CAR-T trials is also associated with high peak CAR-T cells and IL-15 levels, suggesting the need for further investigation into dissociation of efficacy from toxicity (460).

Using expression of chemokines in the tumour microenvironment to guide homing of $CD8^+$ T cells has been explored previously. The Reed-Sternberg cells characteristic of Hodgkin's lymphoma secrete CCL17 and CCL22. Infiltrating $CD8^+$ T cells are infrequently detected in this disease, presumably due to preferential recruitment of T_H2 $CD4^+$ cells and T_{reg} cells expressing CCR4 to create an immuno-suppressive milieu. Genetic modification of $CD8^+$ T cells to over-express CCR4 was able to enhance tumour-specific migration, and combined with tumour antigen-targeted CAR expression mediated enhanced tumour control in mice, but did not alter memory phenotype or enhance effector/target killing beyond that seen using a monocistronic CAR-CD30 vector (461).

Strategies aimed at enhancing T cell homing to particular niches must test the unanticipated effects of altered trafficking. Chemokines such as CXCL12 are also produced in GVHD target organs such as lung, liver and small intestine; while another concern is that various stimuli such as O_2 levels, chemokine signaling or ligand consumption could downregulate the effect of chemokine signaling in specific niches, impairing homing to the desired site, or affecting

BM homeostasis for other immune cells (462). Melanomas, squamous cell and colorectal carcinomas, to choose just three examples, express lower levels of selectins, integrin ligands, and exhibit impaired vascular integrity, reducing T cell homing to the tumour site (463). The chemokine and receptor partner for enhanced T cell homing must therefore be carefully assessed to ensure levels are appropriately high at tumour niches within the target organ, and the site is readily accessible.

1.12.5 Over-expression of CXCR4 in $CD8^+$ T cells

We drew together three important strands of evidence: BM as a unique niche for T cell memory (3, 255, 257, 464), the pivotal role of CXCR4 in BM immune cell homing (53, 281, 282, 284, 465-467) and the finding that CXCR4-deficient $CD8^+$ T cells display impaired homeostatic self-renewal (305). Previous work in my supervisor's lab had generated a retroviral vector enabling constitutive over-expression of CXCR4 through transduction of murine T cells (T^{CXCR4}). Dr. Ben Carpenter demonstrated increased chemotaxis to CXCL12 in vitro and preferential BM accumulation of transferred T^{CXCR4} in irradiated hosts. In a murine model of B-cell lymphoma, allogeneic T^{CXCR4} mediated substantially greater anti-tumour control than allogeneic control T cells ($T^{Control}$) without any worsening of graft versus host disease. Because the tumour stroma in this model expresses CXCL12 (292), one possible mechanism of enhanced control is enhanced T cell homing to the tumour bed. However in vivo imaging demonstrated that $luciferase^+$ T^{CXCR4} did not accumulate at the tumour site earlier or in greater numbers than $T^{Control}$. In addition, T^{CXCR4} did not

outcompete T_{reg} cells to the tumour site, excluding impaired immuno-suppression as an alternative mechanism.

Hypotheses:

Based upon these preliminary findings, I hypothesised that:

a) CXCR4 over-expression increases therapeutic $CD8^+$ T cell homing to BM niches

b) CXCR4 over-expression in therapeutic $CD8^+$ T cells increases the number and functional potential of memory T cells

c) Enhanced memory potential is due to preferential access to cell-extrinsic signaling factors within the BM niche

Aims:

My overall aim for the project was to determine the mechanism underpinning the greater anti-tumour efficacy of T^{CXCR4} through the following strategies:

1. Perform in vivo and ex vivo imaging experiments to identify if T^{CXCR4} home to a specific cellular niche within the BM.

2. Perform a detailed assessment of T^{CXCR4} phenotype and function in vitro and in vivo to identify the mechanism underlying enhanced anti-tumour efficacy using appropriate murine models.

3. Examine the phenotypic effect of altering the duration and level of CXCR4 expression by means of an inducible CXCR4 expression vector.

4. Examine the effect of pharmacological blockade and enhancement of BM homing, and other strategies including physical BM emplacement, upon T cell phenotype.

2 Materials and methods

Mice

C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Margate, UK). *B6.PL-Thy1a/CyJ* (B6 Thy1.1), *B6.SJL-Ptprca Pepcb/BoyJ* (B6 CD45.1), *Flk1-GFP* and Rag1-/- OT-I mice were purchased from the Jackson Laboratory and bred in house. *Rag1ko*, *Rag1ko.IL7ko* and *Rag1ko.IL15rako* mice were a kind gift from Dr Benedict Seddon (UCL, UK). UCL Biological Services bred the above mice in house; irradiated or immune-deficient recipients were maintained in individual ventilated cages. Animals used as recipients for BMT were 10-20 weeks old, and donors were 8-16 weeks old. All procedures were conducted in accordance with the United Kingdom Home Office Animals (Scientific Procedure) Act of 1986, and were approved by the local ethics committees in UCL and Imperial College London (the latter for multi-photon imaging).

Cell lines

Ecotropic Phoenix packaging cells, used for retroviral particle production, were a gift from Dr. G. P. Nolan (Stanford University, USA). The murine A20 B-lymphoblastic cell line has previously been described; in some experiments, the cell line was modified by pMP71 HuCD34 retroviral transduction to express human CD34 (huCD34-A20) followed by isolation and sequential immunomagnetic enrichment using a human CD34 microbead kit (Miltenyi, Germany).

Retroviral vectors, transfection and transduction

The murine *Cxcr4* gene was cloned into a pMP71 retroviral vector by Dr Ben Carpenter to generate pMP71-Cxcr4-IRES-GFP. pMP71-IRES-GFP was used as a control vector (468, 469). Dr Pedro Velica created inducible vectors encoding CXCR4 marked with GFP (pSERS-CXCR4-2A-GFP-M2-Q8), or GFP alone (pSERS-GFP-M2-Q8). Working quantities of plasmid were generated using transformation-competent DH5α bacteria (Invitrogen, Thermo Fisher Scientific, USA). Retroviral transduction was performed as described previously (469). Briefly, Ph-Eco were transfected with relevant plasmid in addition to Fugene-HD transfection reagent (Roche, Switzerland), Opti-MEM serum-free medium and Pcl-Eco DNA. Supernatant medium was used to resuspend $CD8^+$ T-cells in Retronectin-coated (Takara, Japan) non-tissue culture-treated plates. $CD8^+$ T cells were harvested 24 hours prior to transduction and activated with 2 μg/ml concanavalin-A (Sigma-Aldrich, USA) and 1 ng/ml human IL-7 (R&D Systems, USA). Transduction plates were spun at 1000g for 90 minutes and cells were then cultured with T-cell medium and 10 u/ml IL-2 (Roche) on the day of transduction. Medium was refreshed, further IL-2 added on the following day and cells were checked for transduction efficiency at 72 hours post transduction.

Primary and boost vaccination strategy

Isolation of $CD8^+$ T cells or $CD11c^+$ DCs was performed by immunomagnetic selection from splenocytes using Manual MACS® Cell Separation Technology (QuadroMACS Separator, LS columns, CD8a (Ly-2) and CD11c Micro Beads;

Miltenyi, Germany), according to the manufacturer's instructions. For *in vitro* experiments, SIINFEKL peptide (Invitrogen) was added at a concentration of 5 µM. In competitive *in vivo* experiments, T^{CXCR4} and $T^{Control}$ were mixed into a 1:1 ratio prior to injection and vaccination was performed 24 hours and on day 29 by subcutaneous injection of 200 µM SIINFEKL or an irrelevant peptide in a 1:1 ratio with Incomplete Freund's Adjuvant (Sigma-Aldrich, USA). Alternatively, mice received $1x10^6$ $CD11c^+$ peptide-loaded DCs intravenously.

Isolation of murine immune cells

Lymph nodes and spleens: To prepare cell suspensions from spleens and lymph nodes, the freshly removed organs were mashed and passed through a 40 µm cell strainer; red blood cells were removed by isotonic lysis with ammonium chloride (ACK Lysing Buffer; Lonza, Switzerland). Cells were re-suspended in FACS buffer (PBS, 2% FCS, 2 mM EDTA; Lonza) for counting and immunolabelling. Bone marrow: To isolate bone marrow cells, both epiphyses of the long bones of the hind limbs were cut and the bone marrow was flushed out with FACS buffer. The cell suspension was filtered through a 40 µm cell strainer and red blood cells were removed by isotonic lysis with ammonium chloride. Cells were re-suspended in FACS buffer for counting and immunolabelling. Cells were counted using a Neubauer haemocytometer and light microscope. Cell viability was assessed using 0.1% trypan blue (Sigma-Aldrich, USA). $CD8^+$ T cells were isolated by incubation with anti-CD8α MACS beads (Miltenyi, Germany) using LS magnetic columns.

Media and cell culture

T cell medium was derived from RPMI-1640 (Roswell Park Memorial Institute, Lonza, Switzerland) with 10% heat-inactivated foetal bovine serum (FBS, Sigma-Aldrich, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Pen-Strep), 2 mM L-glutamine and 0.05 mM 2-mercaptoethanol. Packaging Phoenix-Eco (Ph-Eco) cell medium was derived from IMDM (Iscove's Modified Dulbecco's Medium, Sigma) with added Pen-Strep and L-glutamine. MACS buffer was derived from phosphate-buffered saline (PBS) with 1% FBS and 0.1% EDTA. Recombinant murine CXCL12, 10 ng/ml IL-15 and SIINFEKL or UTY peptide (5 µM), (all from Peprotech, USA) were added as required.

Flow cytometry

The following monoclonal antibodies were used for flow cytometry: anti-murine CXCR4 (2B11), CD8a (53-6.7), CD62L (MEL-14), CD127 (A7R34), CD122 (TM-β1), CD132 (TUGm2), CD45.2 (104), TNF-α (MP6-XT22), IL-2 (JES6-5H4), BCL2 (3F11) and active caspase-3 (C92-605) (BD Biosciences, USA); anti-murine Thy-1.1 (H1S51), CD44 (IM7), CD25 (PC61), CD45.1 (A20), IFN-γ (XMG1.2), Eomes (Dan11mag), PD-1 (RMP1-30), KLRG1 (2F1) (eBioscience). For intracellular staining, cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, USA). For measurement of proliferation, animals were injected with 100 µg 5-ethynyl-2-deoxyuridine i.p. (EdU) and cells were subsequently stained using the Click-iT Assay Kit (Thermo Fisher Scientific, USA). Multicolour flow cytometry data acquisition

was done with BD LSRFortessa and BD LSR II cell analysers equipped with BD FACSDiva v6.2 software (BD Biosciences, USA). Fluorescence activated cell sorting was performed on a BD FACSAria equipped with BD FACSDiva v5.0.3 software (BD Biosciences, USA). All samples were maintained at 4°C for the duration of the sort. A minimum of 5000 cells was collected and only those with purity ≥ 95% were used for RNA extraction. Cells were sorted directly into Buffer RLT (QIAGEN, USA) with 1% 2-β-mercaptoethanol (Sigma-Aldrich, USA), disrupted through vortexing at 3200rpm for 1min, and immediately stored at -80°C until further processing. Flow cytometry data were analysed with FlowJo X v10 (LLC, USA).

Evaluation of T cell function

In vitro and *ex vivo* analysis of cytokine generation following peptide stimulation was performed as described previously (470). *In vivo* analysis of specific cytotoxicity was performed as described previously (471).

Imaging

Intravital microscopy was performed using a combined Zeiss LSM 780 upright confocal/two-photon microscope as described previously (301). Blood vessels were highlighted by intravenous injection of 8 mg/ml 500 kDa Cy5-Dextran (Nanocs, USA). The following antibodies were used: anti-CXCL12 PE (R&D Systems, USA) and anti-IL15ra (Abcam, UK). For immunofluorescent imaging, femurs and tibias were harvested and processed as described previously

(301). Cryo-preserved sections were re-hydrated in PBS, permeabilized in 0.1% Triton X-100, blocked in 5% goat serum and incubated with primary antibody (goat anti-rabbit IgG Alexa Fluor 633 (Life Tech, Thermo Fisher Scientific, USA) overnight, at 4 °C. After washing in PBS, slides were incubated with secondary antibodies, counter-stained with DAPI (Invitrogen), washed in 0.1% Triton X-100 and mounted using Prolong Diamond Antifade (Invitrogen).

Image quantification

Microscopy data were processed with multiple platforms. Tile scans were stitched using ZEN Black (Zeiss) software. Raw data were visualized and processed using Fiji (472). Automated cell segmentation, distance and volume measurements were performed in Definiens Developer 64 (Delfiniens, Munich, Germany) using local heterogeneity segmentation to isolate $CXCL12^{+}$ cells. Definiens rule sets for these functions are available upon request. Distance measurements from this segmentation were performed as described previously (473). Cell tracking over time was analysed using Imaris (Bitplane, UK).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00 for Mac OsX (GraphPad Software, USA). Significance was assessed using a two-tailed Mann–Whitney U test or two-tailed Wilcoxon signed-rank sum test

for paired comparisons of non-parametric data and a two-tailed student's t-test for parametric data. For multiple comparisons, a one-way ANOVA with Holm-Sidak's post hoc test was used. Survival curve comparison was performed using the log-rank Mantel-Cox test. A *p*-value of ≤0.05 was taken to indicate a significant difference between groups.

3 Defining the BM location of T^{CXCR4}

3.1 Introduction

Polyclonal model

Previous work has established that polyclonal $CD8^+$ T^{CXCR4} show enhanced homing to the bone marrow of irradiated mice in comparison to $T^{Control}$. However CXCL12 expression is upregulated in the context of irradiation (57) and high CXCL12 concentrations have been shown to cause paradoxical repulsion of the receptor-expressing T-cell, known as fugetaxis (474, 475). Accordingly, Dr Pedro Santos e Sousa in our lab conducted short-term competition experiments to exclude the possibility that the homing and persistence characteristics of T^{CXCR4} were being substantially altered by host irradiation.

Equal amounts of polyclonal T^{CXCR4} and $T^{Control}$ were transferred into *Rag1ko* mice. Mice homozygous for the *Rag1*^{tm1Mom} mutation (*Rag1ko*) produce no mature T or B cells and are less likely to reject adoptively transferred T cells expressing a foreign GFP protein. At day 7 post transfer, greater numbers of T^{CXCR4} accumulated in *Rag1ko* mice BM compared to $T^{Control}$ cells (mean T^{CXCR4}: $T^{Control}$ ratio 2.2 ($p=0.026$, two-tailed Mann-Whitney test, $n=10$), as compared to 3.4 for irradiated mice ($p=0.003$) and 10.6 for non-irradiated B6 mice ($p=0.003$) (Figure 3-1A).

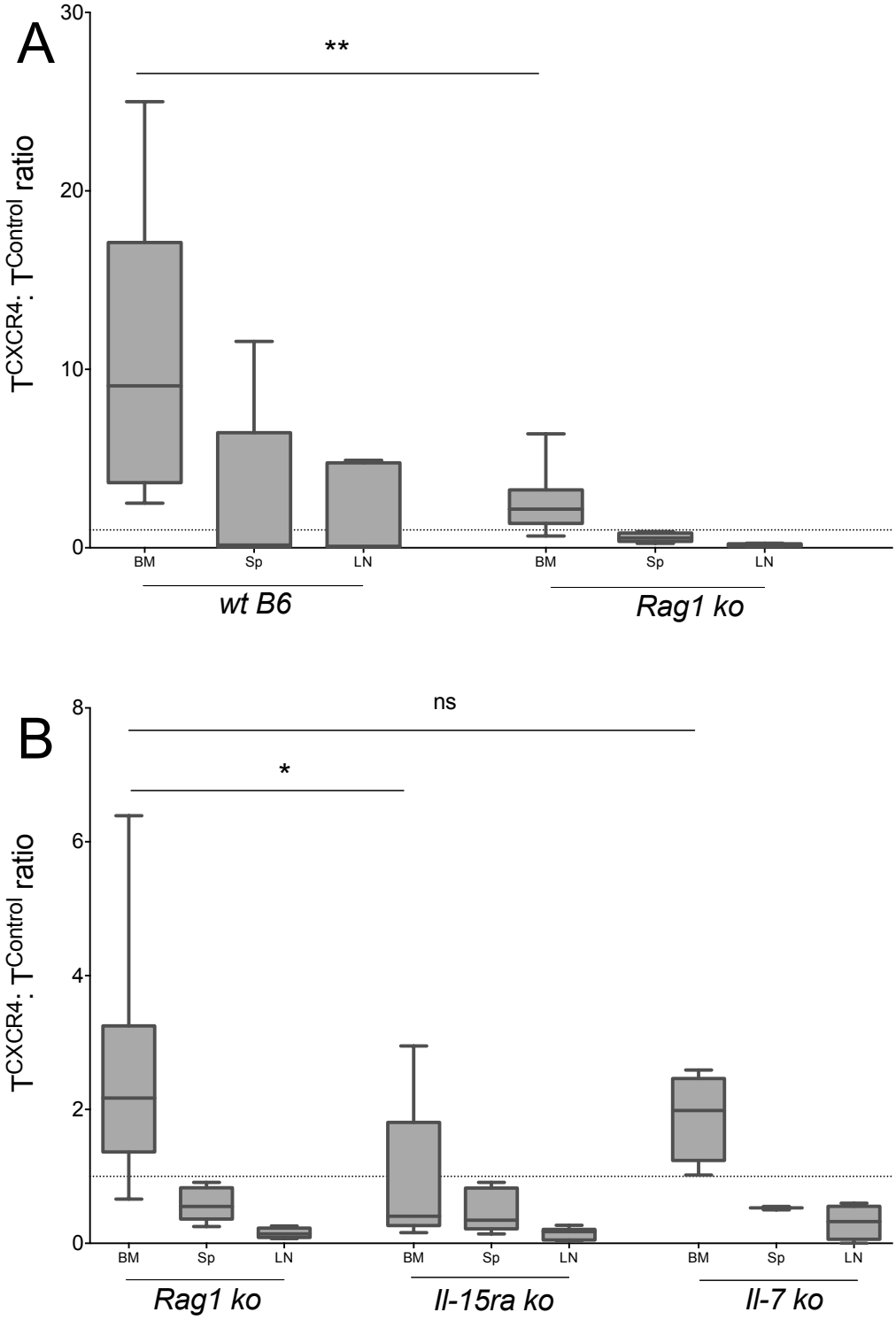


Figure 3-1 Superior T^{CXCR4} BM recruitment is IL-15-dependent.
(A) Box and whisker plots of T^{CXCR4}: $T^{Control}$ ratio in BM, Spleen (Sp) and LN at day 7 following transfer into non-irradiated B6 mice (n=11) and *Rag1ko* mice (n=10). (B) Box and whisker graphs of T^{CXCR4}: $T^{Control}$ ratio in BM, spleen and LN at day 7 following transfer into *Rag1ko* (n=10), *Rag1ko.IL-15rako* (n=10) and *Rag1ko.IL-7ko* (n=4). Data pooled from two independent experiments, *P≤0.05, **P≤0.01.

These experiments showed that T^{CXCR4} retain an advantage in BM homing compared to $T^{Control}$ in a non-inflammatory setting. They also suggest that T^{CXCR4} demonstrate a greater BM homing and retention capacity under non-lymphopenic conditions in non-irradiated B6 mice, ie. when homeostatic cytokines are non-limiting, and hence suggests a competitive advantage in accessing survival factors.

To investigate the importance of access to homeostatic cytokines, similar experiments were performed transferring equal amounts of polyclonal T^{CXCR4} and $T^{Control}$ cells into *Rag1ko* mice genetically deficient in IL-7 (*Rag1ko.IL7ko,* henceforth *IL-7ko mice*) or IL-15Rα (*Rag1ko.IL15rako,* henceforth *IL-15Rako* mice). Strikingly, the advantage in day 7 BM accumulation for T^{CXCR4} was not altered by loss of IL-7 (BM T^{CXCR4}: $T^{Control}$ ratio 2.2 for *Rag1ko* mice vs 2.0 for *Rag1ko.IL7ko* mice, p=ns) but was completely abrogated in the absence of IL-15 signalling (BM T^{CXCR4}: $T^{Control}$ ratio 0.4 for *Rag1ko.IL15rako* mice, p= 0.017 vs *Rag1ko* mice, two-tailed Mann-Whitney test (Figure 3-1B).

Summary

These experiments demonstrated superior BM accumulation of T^{CXCR4} over $T^{Control}$ in a variety of models, particularly in competitive situations where access to homeostatic cytokines is limited. This advantage does not rely upon irradiation but appears to be specifically dependent upon the presence of IL-15Rα. In the light of known expression of CXCL12 and IL-15Rα by stromal cells within the BM (281, 311), I first asked if in vivo imaging could shed light upon these findings by directly identifying T cell interactions within the BM.

3.2 Imaging the BM location of T^{CXCR4}

I first confirmed that expression of CXCR4 was uniformly low in murine B6 bead-sorted $CD8^+$ T cells transduced with pMP71-IRES-GFP (henceforth $T^{Control}$), and that T cells transduced with pMP71-Cxcr4-IRES-GFP (henceforth T^{CXCR4}) expressed CXCR4 in proportion to the level of GFP reporter expression (Figure 3-2).

I considered the possibility that T^{CXCR4} homing to a specific microenvironment in the BM in some way enabled their enhanced antitumour potency and therefore sought to identify the nature of this putative niche. In particular, I aimed to collect detailed information upon T^{CXCR4} interactions with structures within the BM over time, and how this differed from $T^{Control.}$ I therefore performed imaging experiments involving intravenous transfer of polyclonal B6 $CD8^+$ T^{CXCR4} and $T^{Control}$ into separate *Rag1ko* mice, in conjunction with Dr Cristina Lo Celso's group at Imperial College London.

In order to identify the CXCL12-expressing cells, we employed 2 complementary approaches (calvarial intravital multiphoton imaging and immunofluorescence of the long bones). To capture dynamic whole tissue interactions we performed intra-vital calvarial imaging of anaesthetised mice using a custom-built combined fluorescence confocal/ multiphoton microscope at 8 weeks post injection. The extremely thin nature of calvarial bone allows single-cell resolution while the anaesthetic protocol permits detailed tile-based imaging of the entire tissue, and detailed time-lapse microscopy of cell-cell

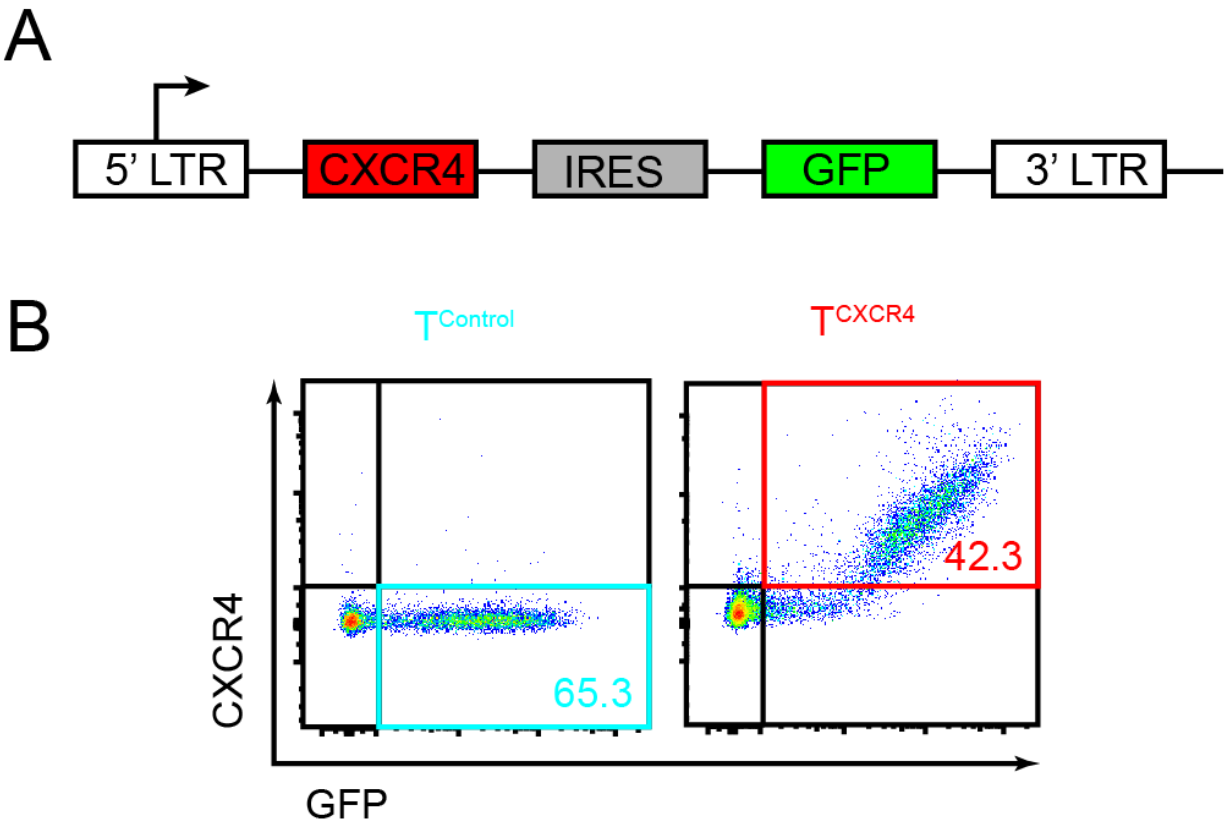
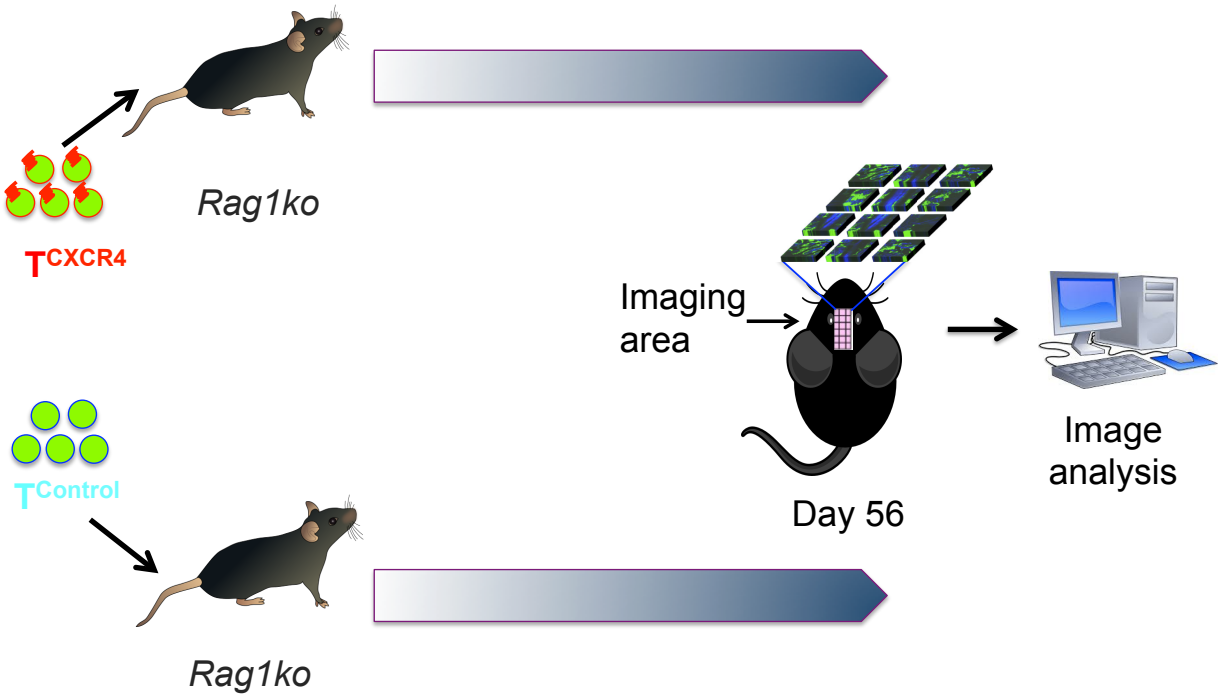


Figure 3-2 Expression of CXCR4 in $T^{Control}$ and T^{CXCR4}.
(A) Schematic depicting CXCR4-GFP retroviral vector map: Long Terminal Repeat (LTR); Internal Ribosome Entry Site (IRES); (B) Representative flow cytometric plots for expression of CXCR4 and GFP in murine B6 $CD8^+$ T cells transduced with the CTRL-GFP vector ($T^{Control}$ in cyan) and CXCR4-GFP (T^{CXCR4} in red), pre-gated upon live lymphocyte gate. Numbers denote % of total $CD8^+$ T cells.

interactions. A surgically implanted imaging window using a lock and key mechanism was used to ensure accurate positioning of the mice on the microscope without motion artefact (476) (Figure 3-3).

The calvarium has been shown to be equivalent to the long bones with regards to haematopoietic cell frequency, function and localisation, and allows longitudinal imaging with minimal intervention (477, 478). We performed immuno-imaging to define CXCL12 localisation by intravenous injection of

fluorescent antibodies to label cell-surface bound chemokine, between 5 and 60 minutes before imaging (479). Mature T cells are known to localize to specialized vascular subregions within the BM (479) and we therefore identified vascular structures by means of iv injection of 500 kDa Cy5-Dextran (301). Raw microscopy data were then processed using local heterogeneity segmentation (LH-SEG) on the Definiens platform to isolate $CXCL12^{+}$ cells and perform unbiased 3D distance measurements between cellular BM elements (473). Cell tracking over time was performed using Imaris (Bitplane, UK) to identify speed and motion characteristics of T cells.



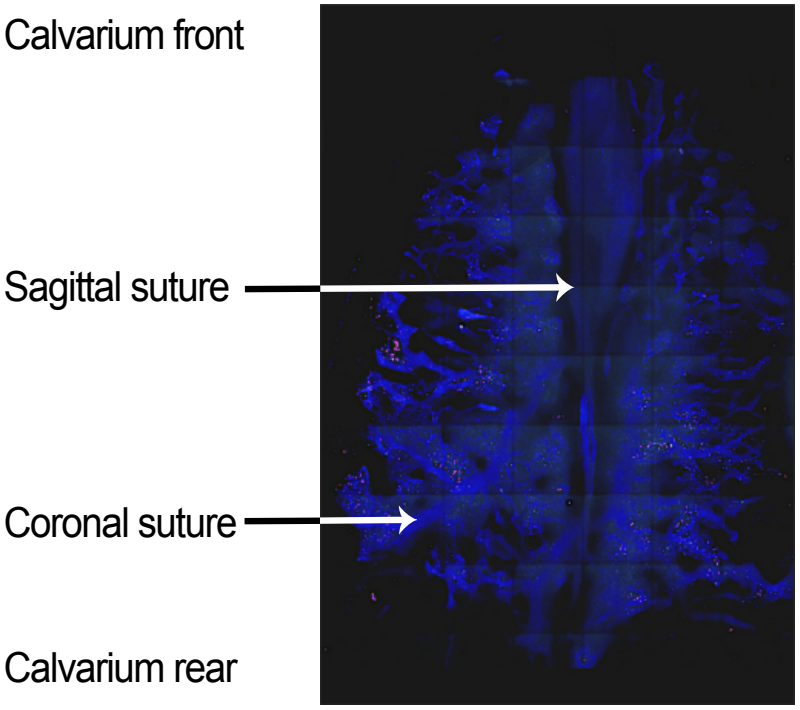
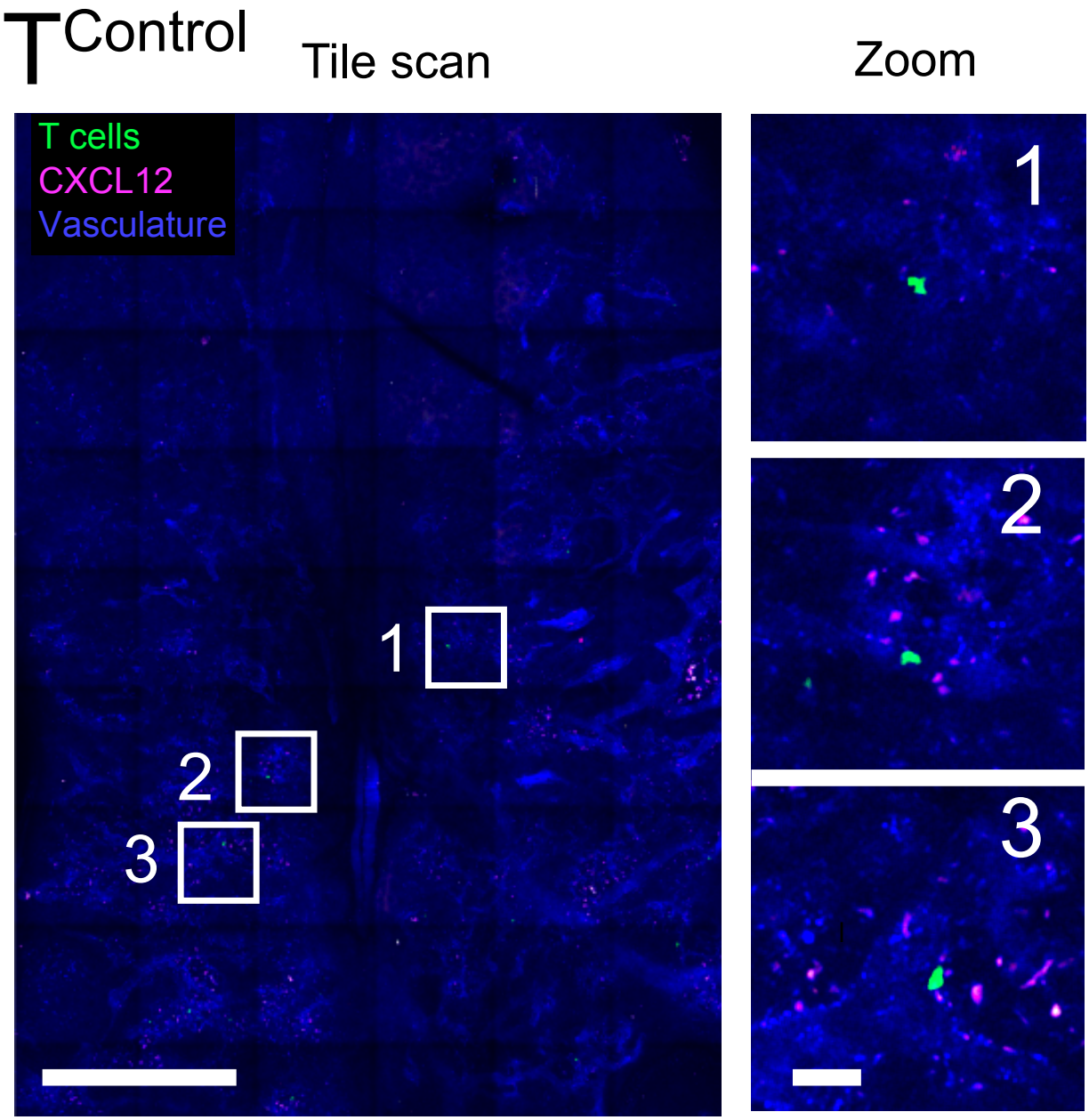


Figure 3-3 Experimental approach to in vivo BM imaging.
Rag1ko mice were anaesthetised 8 weeks post injection of T^{CXCR4} or $T^{Control}$. Tile scan imaging of multiple *z*-stacks was performed, enabling single-cell time-lapse imaging of T cells within the whole calvarial space, following iv injection of CXCL12 PE and Cy5-dextran antibody.

Visual evaluation of T^{CXCR4} location within the BM

Consistent with flow cytometric findings, T^{CXCR4} were more numerous than $T^{Control}$ in the bone marrow for all mice imaged (Figure 3-4). T^{CXCR4} associated in dense clusters of cells close to vascular structures, whereas $T^{Control}$ appeared as single cells with less obvious proximity to vascular cells, as seen in the zoom insets. The distribution of CXCL12 staining was predominantly in close association with vascular structures.



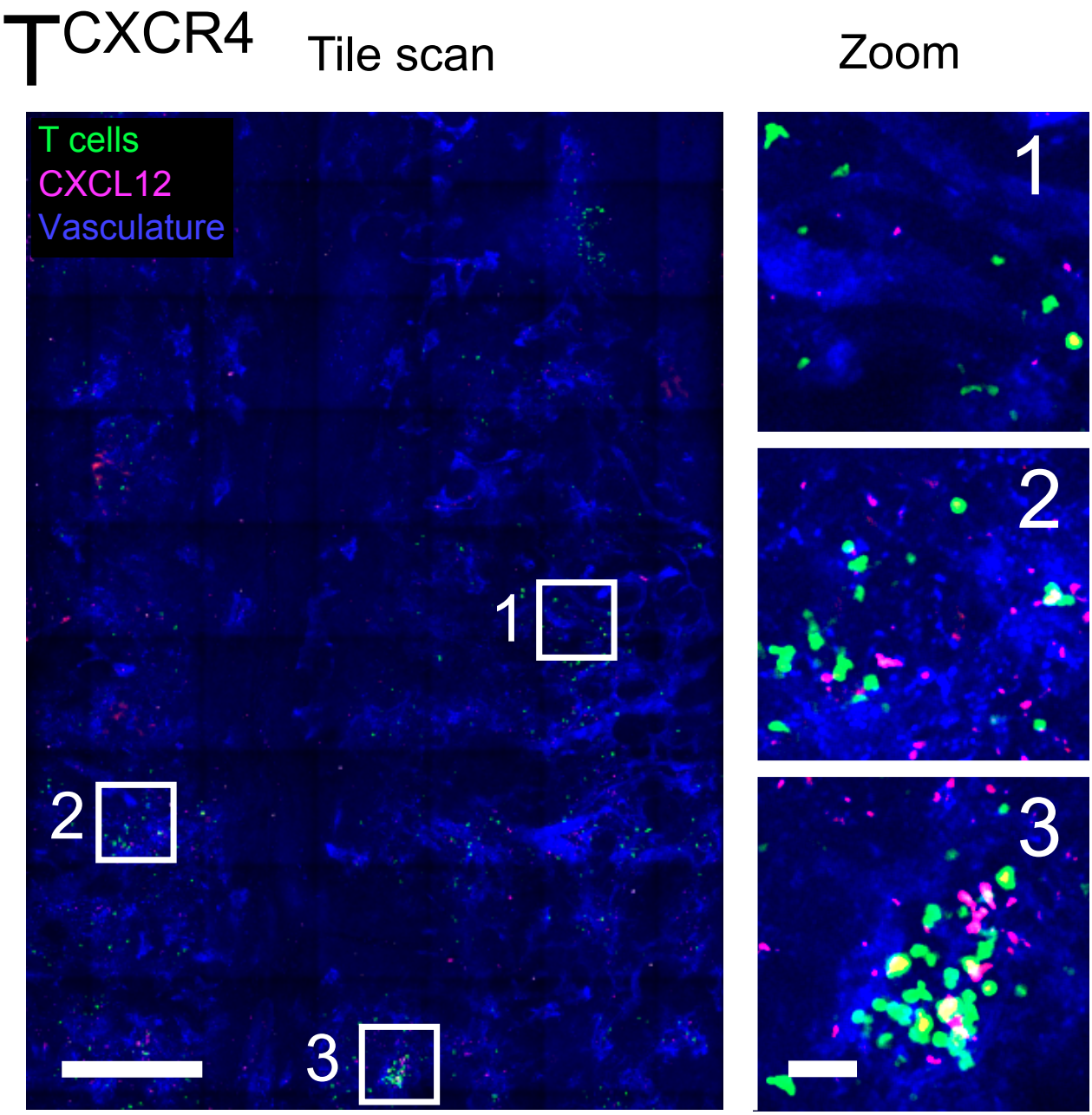


Figure 3-4 T^{CXCR4} accumulate in greater numbers in perivascular BM locations.

Representative maximum projection tile scans and corresponding high magnification zoom insets for intra-vital calvarial imaging of transduced $T^{Control}$ (top) and T^{CXCR4} (bottom), both in green, in proximity to $CXCL12^{+}$ cells (purple) and vasculature (blue). Scale bars: 500 µm in tile scan image, 50 µm in zoom images.

Association with calvarial CXCL12^{+} cells

We next sought to quantitate the distance of each T cells from the CXCL12-producing cells. We performed image analysis on cells, using GFP fluorescent intensity to evaluate the level of CXCR4 expression in T^{CXCR4}. We compared T^{CXCR4} cells with a GFP intensity >25 ($T^{CXCR4\ HIGH}$) with $T^{CXCR4\ LOW}$ (GFP intensity 10-15) to test competition between cells expressing different levels of CXCR4 in the same environment, the latter acting as an internal control. Significantly more $T^{CXCR4\ HIGH}$ cells were located within 0-5 μm of the CXCL12^{+} cells than $T^{CXCR4\ LOW}$ (48% vs 22%, Mann-Whitney test $p<0.0001$) and 12% of $T^{CXCR4\ HIGH}$ were in direct contact with CXCL12-expressing cells compared to 2% of $T^{CXCR4\ LOW}$ ($p=0.015$) (Figure 3-5A and B). The median distance of all $T^{CXCR4\ HIGH}$ from CXCL12-expressing cells was 5.3 μm vs 9.8 μm for $T^{CXCR4\ LOW}$ ($p<0.0001$).

Next, we asked whether CXCR4 over-expression enabled T^{CXCR4} to home more effectively to CXCL12^{+} cells than $T^{Control}$. We found that significantly more $T^{CXCR4\ HIGH}$ cells were located within 0-5 μm of the CXCL12^{+} cells than $T^{Control}$ (48% vs 32%, Mann-Whitney test $p<0.0001$) and a trend for greater numbers of T^{CXCR4} were in direct contact with CXCL12^{+} cells compared to $T^{Control}$ (12% vs 2%, $p=0.070$) (Figure 3-5C and D). $T^{CXCR4\ HIGH}$ were located closer to the CXCL12+ cells compared to $T^{Control}$ (median distance 5.3 vs 8.5 μm, $p=0.026$). The level of CXCR4 expression on T^{CXCR4} inversely correlated with distance to the nearest CXCL12^{+} cell for T^{CXCR4} but not $T^{Control}$ (distance from CXCL12^{+} cells considered as a continuous function, Figure 3-5E).

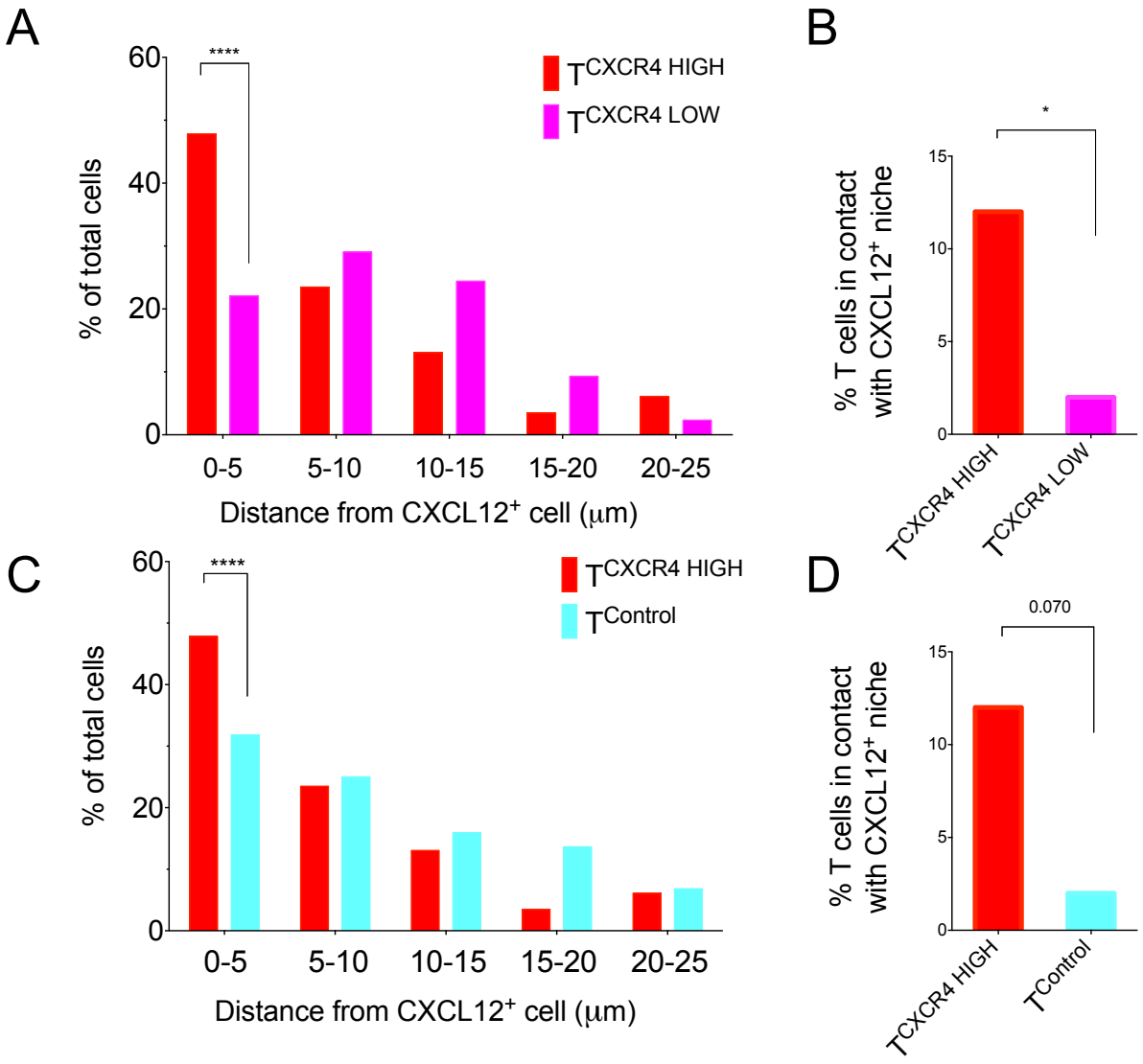

A
B
C
D
E
% of total cells
Distance from CXCL12+ cell (μm)
TCXCR4 HIGH
TCXCR4 LOW
TControl
****
*
0.070
% T cells in contact with CXCL12+ niche
0-5
5-10
10-15
15-20
20-25
r = -0.157
p = 0.256
r = -0.329
p < 0.001
GFP fluorescence (a.u.)

Figure 3-5 High CXCR4 expression dictates location in relation to calvarial CXCL12-expressing cells

(A) Frequency distribution histogram representing proximity of $T^{CXCR4\ HIGH}$ (red) and $T^{CXCR4\ LOW}$ (purple) to calvarial $CXCL12^{+}$ cells. (B) Proportion of total $T^{CXCR4\ HIGH}$ and $T^{CXCR4\ LOW}$ in contact with the $CXCL12^{+}$ cells. (C) and (D) As above for $T^{CXCR4\ HIGH}$ and $T^{Control}$ (cyan), *P≤0.05, ****P≤0.0001. (E) X-y graphs comparing GFP intensity vs. distance of individual T^{CXCR4} and $T^{Control}$ from $CXCL12^{+}$ cells measured on static images derived from the same experiments. Inset denotes Pearson's correlation coefficient *r* and significance. n=2 $T^{Control}$ and 3 T^{CXCR4}, 1 independent experiment.

Long-bone T^{CXCR4} location

We then tested if the T^{CXCR4} relationship with CXCL12-expressing cells was replicated using a different experimental strategy. Following intravenous transfer of polyclonal B6 $CD8^{+}$ T^{CXCR4} and $T^{Control}$ into separate *Rag1ko* mice, we performed immunofluorescence of femur and tibia sections using anti-CXCL12 antibody.

Similar to the calvarial model, we found that a significantly higher proportion of $T^{CXCR4\ HIGH}$ were located within 0-5 μm of $CXCL12^{+}$ cells than $T^{CXCR4\ LOW}$ (62% vs 32%, p=0.008) and greater numbers of $T^{CXCR4\ HIGH}$ were in direct contact with $CXCL12^{+}$ cells compared to $T^{CXCR4\ LOW}$ (25% vs 6.4%, p<0.0001) (Figure 3-6A and B). Furthermore, we found that a significantly higher proportion of $T^{CXCR4\ HIGH}$ were located within 0-5 μm of $CXCL12^{+}$ cells than $T^{Control}$ (62% vs 41%, p=0.003) and greater numbers of $T^{CXCR4\ HIGH}$ were in direct contact with $CXCL12^{+}$ cells compared to $T^{Control}$ (25% vs 6.6%, p<0.0001) (Figure 3-6C and D).

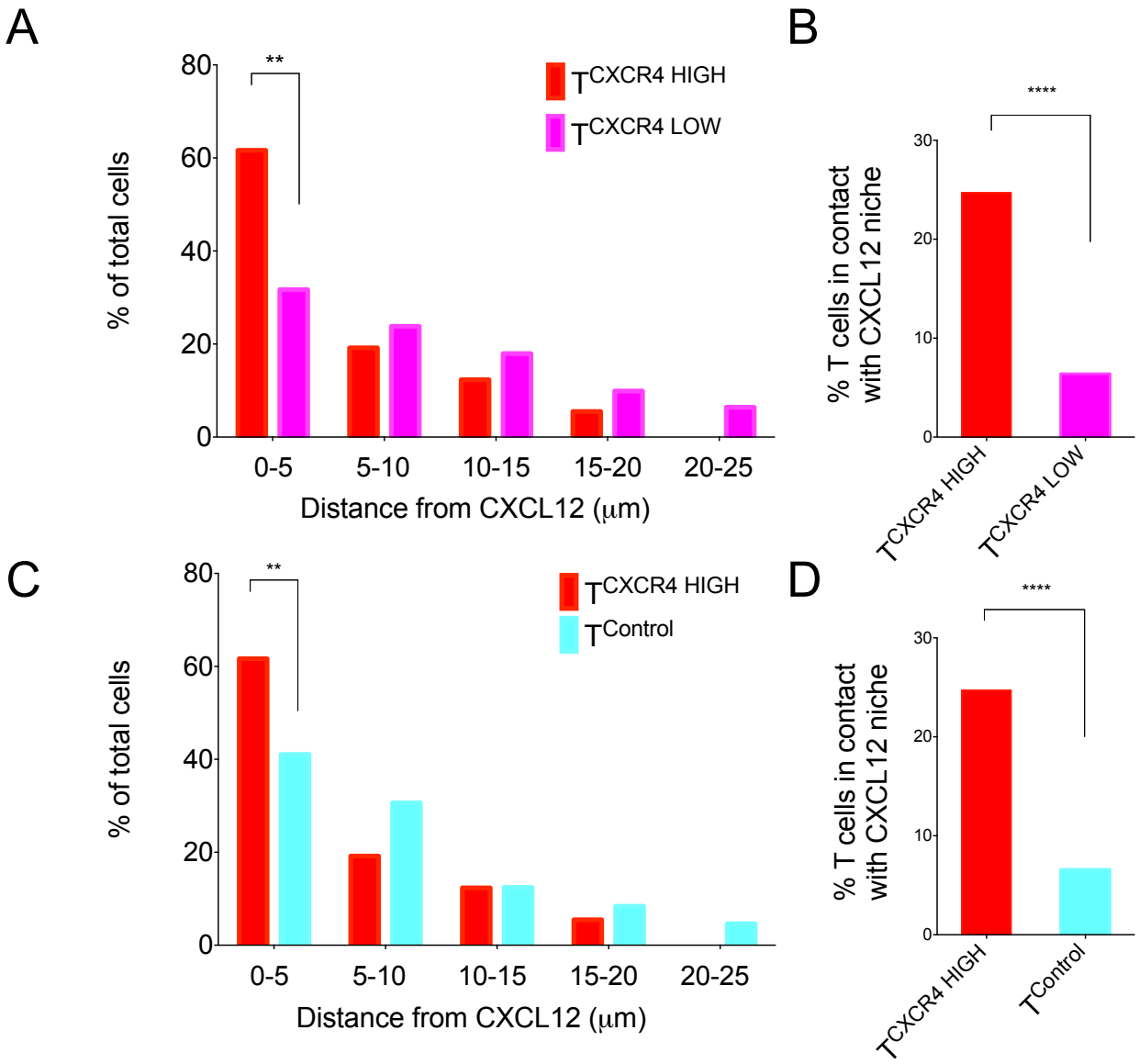


Figure 3-6 Over-expression of CXCR4 enables closer localisation to CXCL12^{+} cells in long bones.

(A) Frequency distribution histogram representing proximity of $T^{CXCR4\ HIGH}$ (red) and $T^{CXCR4\ LOW}$ (purple) to long bone CXCL12^{+} cells. (B) Proportion of total $T^{CXCR4\ HIGH}$ and $T^{CXCR4\ LOW}$ in contact with CXCL12^{+} cells. (C) and (D) As above for $T^{CXCR4\ HIGH}$ and $T^{Control}$ (cyan). n=2 $T^{Control}$ and 2 T^{CXCR4}, 1 independent experiment, **P≤0.01, ****P≤0.0001.

T^{CXCR4} dynamic motion

T cell motility and antigen scanning behaviour is modulated by chemokine cues within lymphoid environments, including CXCL12 (27, 480). I therefore assessed whether T^{CXCR4} moved differently compared to $T^{Control}$ within the calvarial BM, using Imaris software to track multiple individual cells in 3 dimensions over 30 minute time periods. Both T^{CXCR4} and $T^{Control}$ cells displayed substantial variation in dynamic behaviour. Some cells appeared to be arrested and largely retained a similar shape and position throughout the imaging period, often in close association with $CXCL12^{+}$ cells, whilst other cells moved dynamically through the bone marrow environment, elongating into a polarized shape and altering speed as they mobilised around the physical parenchyma, often appearing to physically scan the surface of other cells (481). Videos available from: https://www.jci.org/articles/view/97454/sd/4 https://www.jci.org/articles/view/97454/sd/5 https://www.jci.org/articles/view/97454/sd/6 https://www.jci.org/articles/view/97454/sd/7.

Perivascular CXCL12 expression is known to affect lymphocyte rolling and movement within secondary lymphoid organs (23, 26), and this effect is likely to be heightened in cells expressing high levels of CXCR4. As GFP brightness indicates the level of CXCR4 expression in T^{CXCR4} but not in $T^{Control}$, I was able to directly examine the effect of CXCR4 expression on T cell speed. T cells moving very quickly through the circulation without adhering to BM parenchyma or vascular structures were not included in the analysis. GFP intensity correlated positively with maximum T cell speed for T^{CXCR4} cells but not for $T^{Control}$ (Spearman's *rho* = 0.46 vs 0.29, p=0.011 for T^{CXCR4}, ns for

$T^{Control}$) and to a lesser extent for mean speed (Spearman's *rho* = 0.34 vs 0.13, p=0.067 for T^{CXCR4}, ns for $T^{Control}$).

I measured another aspect of dynamic motion over time by calculating the arrest coefficient (AC) for each cell, using the standard definition of the proportion of time with a speed <2 μm/minute. We found that T^{CXCR4} cells could be divided into 2 distinct populations (Figure 3-7A): those with a high (Arrested, AC>0.75) and those with a low arrest coefficient (Migratory, AC<0.75). This clear difference was not evident in the $T^{Control}$ population. Furthermore, I found that migratory T^{CXCR4} moved twice as fast as migratory $T^{Control}$ cells (mean speed 0.14 μm/s vs 0.07 μm/s, Mann-Whitney p=0.003). Accordingly, mean velocity of all T^{CXCR4} was greater than $T^{Control}$ (3.9 ± 3.3 μm/min vs 2.2 ± 1.6 μm/min, p=0.004 (Figure 3-7B). We did not see a difference in arrest coefficients between $T^{CXCR4\ HIGH}$ and $T^{CXCR4\ LOW}$ cells and speed of migratory cells was similar (median 0.14 vs 0.12 μm/s, p=ns). When analysing the pattern of movement, I found that the speed of T^{CXCR4} varied significantly less than $T^{Control}$, ie. T^{CXCR4} tended to remain either migratory or arrested (median speed variation 0.6 vs 0.95, p<0.001, speed variation defined as standard deviation of speed) and their tracks were significantly less straight (median track straightness 0.17 vs 0.38, p=0.004, straightness defined as displacement/length). $T^{CXCR4\ HIGH}$ cells also moved in less straight tracks than $T^{CXCR4\ LOW}$ (0.17 vs 0.27, p=0.038). This deviation from straight tracks is presumably due to molecular cues, for which CXCL12 is a prime candidate.

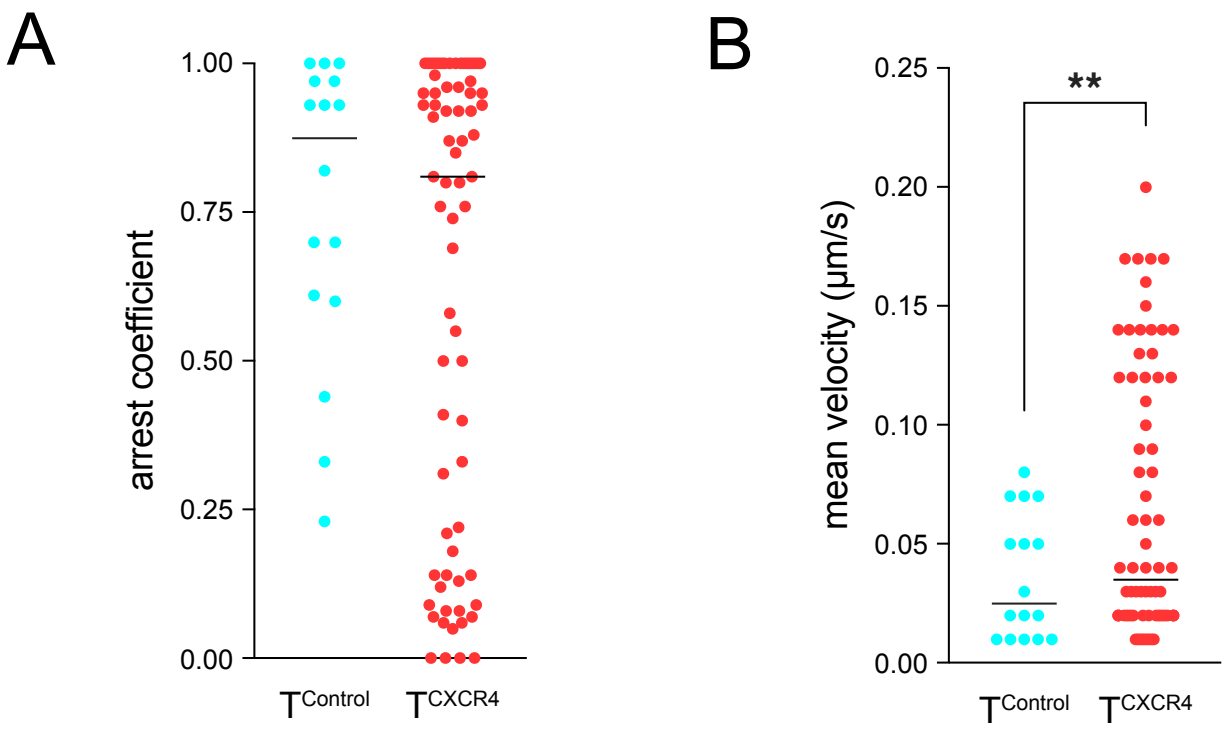


Figure 3-7 CXCR4 over-expression alters migratory behavior.
Summary data showing (A) arrest coefficient (AC) and (B) mean velocity data for time lapse imaging of T^{CXCR4} and $T^{Control}$). Median tracking period was 8.5 min/cell, range 8.5-30 min/cell (total number of cells tracked: n=72 T^{CXCR4}, n=16 $T^{Control)}$. Data pooled from 14 different regions of interest in 5 mice, n=2 $T^{Control}$ and 3 T^{CXCR4}, 1 independent experiment, data, **P≤0.01.

Defining the perivascular CXCL12$^+$ population

I then investigated whether the vascular CXCL12$^+$ cell populations seen in the calvarial model were of endothelial origin or other CXCL12 sources, such as perivascular mesenchymal stromal cells. I therefore injected iv CXCL12-PE antibody and Cy5-dextran into *Flk1*-GFP mice where GFP expression is confined to endothelial cells and performed intra vital calvarial imaging. As seen in the magnified composite images in Figure 3-8, the vascular CXCL12$^+$ cells associated with T^{CXCR4} in the calvarial model were primarily of endothelial origin (yellow) but some sources of CXCL12 were of non-endothelial origin (purple). This conclusion is dependent upon the imaging model used and delivery of CXCL12 staining antibody via the intravenous route, which may alter staining characteristics.

I therefore attempted to further define the CXCL12$^+$ cells with which the T^{CXCR4} were interacting. CD8$^+$ T cells in the BM are maintained by IL-15, trans-presented on stromal cells also expressing IL-15Rα (482). The primary source of IL-15 in the BM matches the phenotype of CXCL12-abundant reticular cells (311) and we therefore used complementary imaging models to ask if T^{CXCR4} localise preferentially to IL-15Rα$^+$ cells.

I also used the calvarial imaging model to ask if IL-15Rα$^+$ cells were associated with vascular structures by injecting IL-15Rα antibody iv followed by secondary Alexa Fluor 633 label; however the resultant images did not identify any positive staining, which may relate to technical issues with the antibody, impaired extravascular delivery due to the intravenous route or low IL15Rα$^+$ expression levels in the non-inflammatory setting (311, 483).

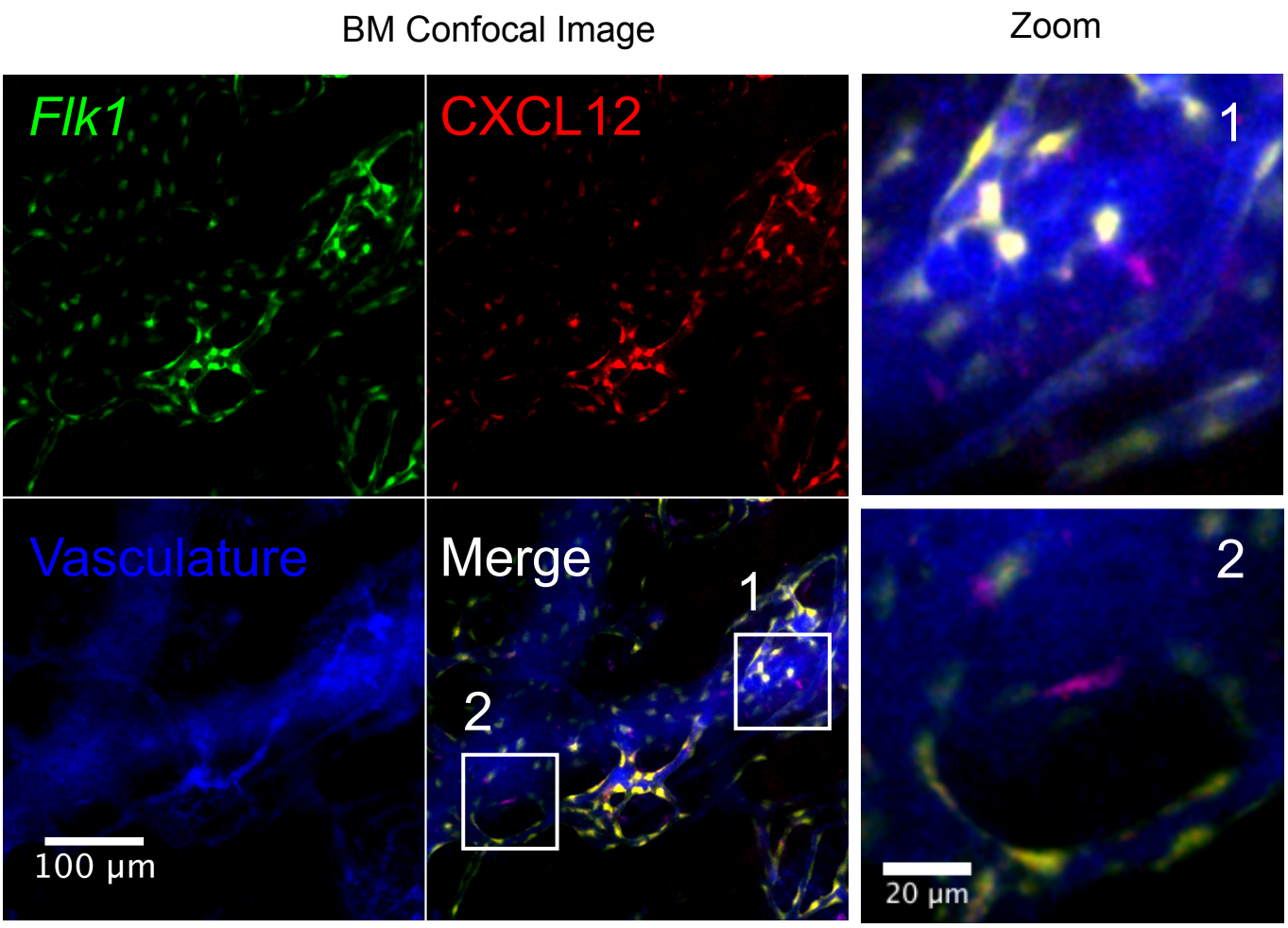


Figure 3-8 Vascular CXCL12^{+} cells in the BM are of both endothelial and non-endothelial origin.
Intra-vital confocal imaging was performed on *Flk1*-GFP mice. Calvarial images and high magnification insets are shown following intravenous injection of anti-CXCL12-PE (red) and Cy5-dextran to identify vasculature (blue). Yellow identifies endothelial sources of CXCL12 and purple non-endothelial sources.

We therefore moved to the immunofluorescent long bone model and stained sections with an IL-15Rα antibody and secondary Alexa Fluor 633 label. We otherwise used the same experimental model as before, injecting T^{CXCR4} and $T^{Control}$ into separate *Rag1ko* mice and quantitating distances to IL-15Rα^{+} cells. In a competitive setting we found that significantly more $T^{CXCR4\ HIGH}$ cells were

located within 0-5 µm of IL-15Rα^{+} cells than T$^{CXCR4\ LOW}$ (57% vs 26%, Mann-Whitney test p<0.0001) and 24% of T$^{CXCR4\ HIGH}$ were in direct contact with IL-15Rα^{+} cells compared to only 6% T$^{CXCR4\ LOW}$ (p<0.0001) (Figure 3-9A and B).

Next, we compared distances with mice receiving T^{Control} cells, ie. not in direct competition with T^{CXCR4} cells. In contrast to the CXCL12 setting, we found that only slightly more T$^{CXCR4\ HIGH}$ cells were located within 0-5 µm of the IL-15Rα^{+} expressing cells compared to T^{Control} (57% vs 54%, p=0.017); or were in direct contact with IL-15Rα^{+} cells compared to T^{Control} (24% vs 19%, p=0.016) (Figure 3-9C and D).

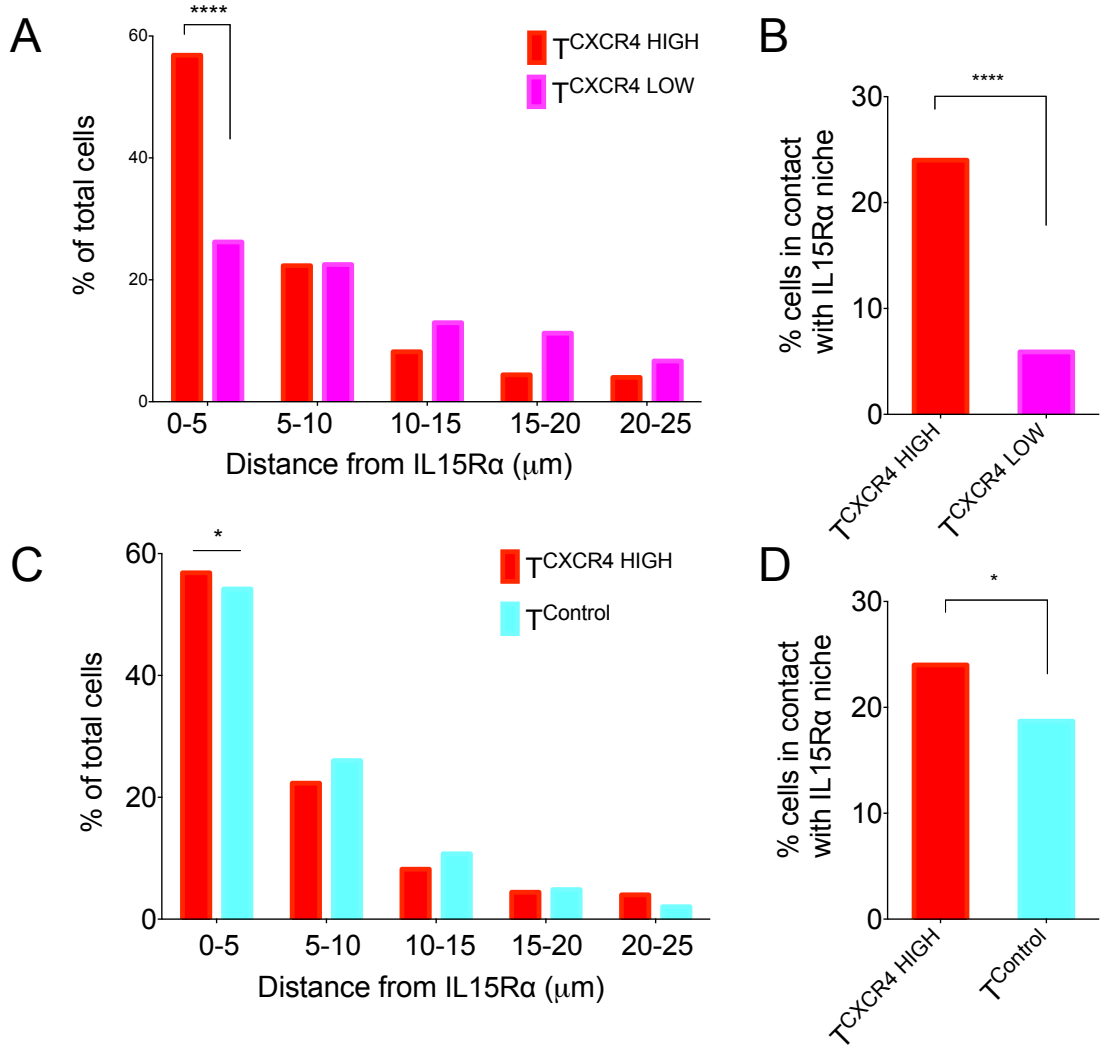


Figure 3-9 CXCR4 expression level dictates access to IL-15.
(A) Frequency distribution histogram representing proximity of $T^{CXCR4\ HIGH}$ (red) and $T^{CXCR4\ LOW}$ (purple) to long bone IL-15Rα$^+$ cells. (B) Proportion of total $T^{CXCR4\ HIGH}$ and $T^{CXCR4\ LOW}$ in contact with IL-15Rα$^+$ expressing cells (C) and (D) As above for $T^{CXCR4\ HIGH}$ and $T^{Control}$ (cyan), n=2 $T^{Control}$ and 2 T^{CXCR4}, 1 independent experiment.

3.3 Discussion

These experiments directly visualised the clustering association of T^{CXCR4} within the bone marrow microenvironment, in close proximity to vascular structures. Increased CXCR4 expression enhanced proximity to and direct contact with CXCL12-expressing cells in two different experimental models and anatomical locations. T^{CXCR4} also moved faster than $T^{Control}$ and contained a subset of highly motile cells with low arrest coefficients, suggesting that higher CXCR4 expression is responsible for this behaviour and close association with vascular-associated CXCL12$^+$ cells. We did not see a different in migratory speed between $T^{CXCR4\ HIGH}$ and $T^{CXCR4\ LOW}$ cells, This may be due to the fact that over a certain threshold of CXCR4 expression, speed may not increase, or that higher CXCR4 expression may have counter-acting effects on speed by enhanced attraction to stationary CXCL12 sources. This theory is supported by the reduction in track straightness seen in cells expressing progressively higher levels of CXCR4.

The nature of the CXCL12$^+$ cells interacting with T^{CXCR4} is still up for debate as the Flk1$^+$ model showed that intravenously injected CXCL12 antibody stains a substantial proportion of endothelial cells. In conjunction with observation of the dynamic behaviour of extravascular T^{CXCR4} within the BM parenchyma and studies showing that perivascular stromal cells express 125-

fold higher levels of CXCL12 than endothelial cells (281) this may indicate a limitation of the intravenous antibody model. Antibody diffusion kinetics or staining characteristics may cause preferential uptake of antibody by CXCL12 on the lumenal surface of vasculature, preventing an accurate tissue-wide view of CXCL12 distribution.

In support of this concept, long bone immunofluorescence showed that in a competitive situation, cells expressing high levels of CXCR4 outcompete those expressing lower levels ($T^{CXCR4\ LOW}$) to be in contact with IL-15Rα$^+$ cells. This difference was not noted between T^{CXCR4} and $T^{Control}$ cells in separate mice. We therefore draw the conclusion that if higher CXCR4 expression allows cells to compete better in accessing an IL-15Rα$^+$ microenvironment, the sources of CXCL12 and IL-15/IL-15Rα may overlap. This may be a valuable advantage in situations where access to trans-presented IL-15 is limiting, or multiple other cells are competing for these niches. Given the limited current range of IL-15Rα antibodies, however, coupled with lower expression in the non-inflamed situation, other models are required to test these conclusions.

Further imaging experiments were not performed due to lack of appropriate experimental tools and time, but would ideally employ IL-7 & IL-15 reporter mice in conjunction with stromal reporter strains to dissect the nature and behaviour of this microenvironment in detail (282, 311). Specifically, it would be important to evaluate how T^{CXCR4} behaviour might change in response to an inflamed or tumour microenvironment. Enhanced motility would enhance the chances of encounter with dendritic cells and immuno-stimulatory

cytokines, but might also reduce the duration of antigen priming with implications for memory formation (484).

4 The effect of CXCR4 overexpression on memory differentiation

4.1 Introduction

Based upon this evidence that T^{CXCR4} preferentially home to a $CXCL12^{+}$ microenvironment in the BM, I considered whether this finding might be responsible for preferential anti-tumour efficacy and a previously observed tendency to retain CD62L expression. In order to investigate the mechanism underlying the memory phenotype associated with T^{CXCR4}, and whether it might be responsible for enhanced functionality, I then developed an in vivo vaccination model to study antigen response and the influence of homeostatic cytokines in the BM in more detail.

I first magnetic bead-sorted splenic OT-I CD8+ T cells, which express a TCR specific for the SIINFEKL peptide (485), and transduced them with either CXCR4-GFP or CTRL-GFP according to congenic marker. The use of vaccination allows controlled induction of a memory phenotype with observation of the kinetics of expansion and reduction in the population over time, in addition to markers of memory and cytokine receptors.

In the first competition experiment, transduced T-cells were mixed in a 1:1 ratio of T^{CXCR4}: $T^{Control}$ and transferred iv into non-irradiated wild type B6 mice to assess a steady state response in the absence of the complicating factor of irradiation. Mice were vaccinated with bone marrow-derived dendritic cells pulsed with relevant (SIINFEKL) or irrelevant (UTY) peptide on day 1, and

harvest of bone marrow, spleen, peripheral blood and lymph nodes was performed on days 8 and 29 post injection. Mice receiving UTY peptide are henceforth referred to as unvaccinated.

I observed enhanced bone marrow accumulation of T^{CXCR4} compared to $T^{Control}$ (median ratio 6.8:1, n=2) at day 8 in vaccinated mice. Insufficient GFP positive cells were seen in unvaccinated mice; however, at day 29 no GFP expression was seen for any animals in any organs. One explanation for this finding is that GFP-expressing cells, identified as bearing an immunogenic foreign protein, had been deleted by an intact host immune system response (486). Another possibility is that competition with endogenous lymphocytes had prevented adoptively transferred cells from engrafting in sufficient numbers in occupied niches, preventing their access to scarce survival factors such as homeostatic cytokines. This latter possibility is less likely because reasonable numbers of T cells were observed to be persisting in bone marrow and splenic environments at the early time point post SIINFEKL vaccination; however, the low numbers of cells observed in mice vaccinated with irrelevant peptide suggest that antigen-inexperienced cells may not persist long term in this model without access to adequate cytokine support.

I therefore explored a model where the absence of lymphocytes acting as cytokine sinks reduces competition between adoptively transferred cells for survival and proliferation factors. I modified the vaccination model to use *Rag1ko* mice as recipients. Their deficiency in the recombination-activating gene prevents formation of mature T or B cells; however, innate immune cells including NK cells persist. This lymphopenic environment mimics the clinical

environment post bone marrow transplantation, and chemotherapy as used in current immunotherapy trials, with relevance to translation.

I modified the vaccination protocol to incorporate primary and boost vaccinations with subcutaneous peptide pre-mixed with Incomplete Freund's Adjuvant (IFA) at days 1 and 29. This strategy is associated with a robust antigen-specific response and the use of IFA plus antigen provides an antigen depot permitting prolonged antigen uptake and presentation in vivo by dendritic cells.

In order to monitor the kinetics of the peak primary and boost vaccination responses, and compare them with a 'steady state' phenotype, I harvested secondary lymphoid organs from recipients at peak of primary vaccination (day 8), at day 29 post-primary response and following boost (day 36). These time points were chosen based upon well-established vaccination models of T cell memory utilising OT-I T cells, where assessment 7 days post primary and secondary infection defined distinct differences in T cell phenotype and clonal expansion that persisted over time (487). Splenocytes were re-stimulated with cognate peptide ex vivo to measure functional cytokine production (see Figure 4-1 for model). T cells were left without exposure to antigen for 4 weeks between primary and secondary vaccination, and analysed prior to secondary vaccination (steady state) to identify changes in the initial phenotype corresponding to homeostatic cytokine exposure.

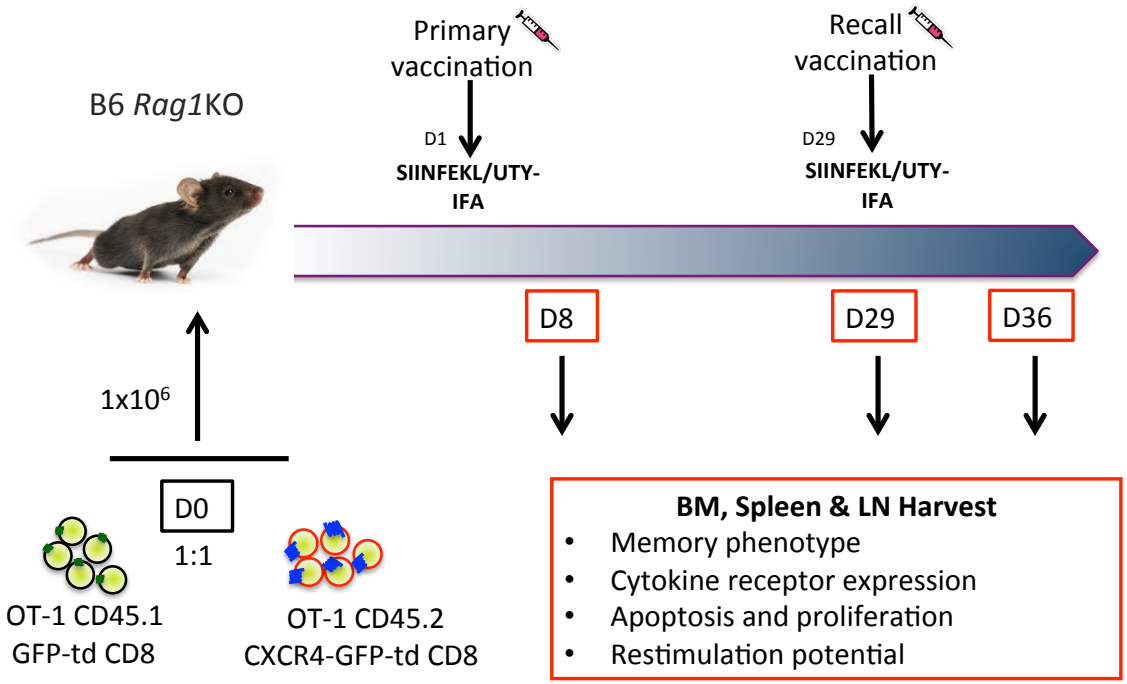


Figure 4-1 Constitutive vaccination model.
Equal numbers of OT-I T^{CXCR4} and $T^{Control}$ were co-injected into *Rag1ko* mice, prior to vaccination with relevant SIINFEKL or irrelevant UTY peptide at days 1 and 29. Tissues were harvested at days 8, 29 and 36 to examine accumulation and phenotype.

4.2 Primary constitutive vaccination response

On the day of injection (day 0), T^{CXCR4} and $T^{Control}$ were comparable in terms of median T^{CM} % (64 vs 53, 2 tailed t-test; p=ns), CD62L, CD44 and cytokine receptor expression.

Number and ratio

At day 8 in vaccinated mice receiving SIINFEKL antigen, T^{CXCR4} demonstrated an advantage in terms of absolute number and ratio in BM and spleen, as shown in Figure 4-2 and Figure 4-3 below. Median BM T^{CXCR4} cell number was 26462 vs 1269 $T^{Control}$, spleen T^{CXCR4} 20740 vs 10355 $T^{Control}$ (2 tailed paired t test; p=0.050 and ns respectively). Lymph node numbers were similar (5,198 vs 5,812). T^{CXCR4}: $T^{Control}$ ratio was dramatically higher in bone marrow (median 14.4:1, 2 tailed paired t-test; p=0.03), higher in spleen (median 2.2:1, p=ns) and similar in lymph node (0.85:1, p=ns). The numerical advantage was notably higher for mice not exposed to relevant antigen (BM median ratio 52.7:1, spleen 6.8:1, lymph node 1.6:1, p=0.07, ns, ns respectively). Similarly, median BM T^{CXCR4} cell number was 19,651 vs 462, spleen 15,475 vs 1355, lymph node 2576 vs 1559. These data show greater accumulation of T^{CXCR4} cells in key secondary lymphoid organs, particularly in the absence of relevant antigen. In fact, T^{CXCR4} maintain higher numbers in unvaccinated spleen and BM than $T^{Control}$ undergoing antigen-dependent proliferation in vaccinated mice.

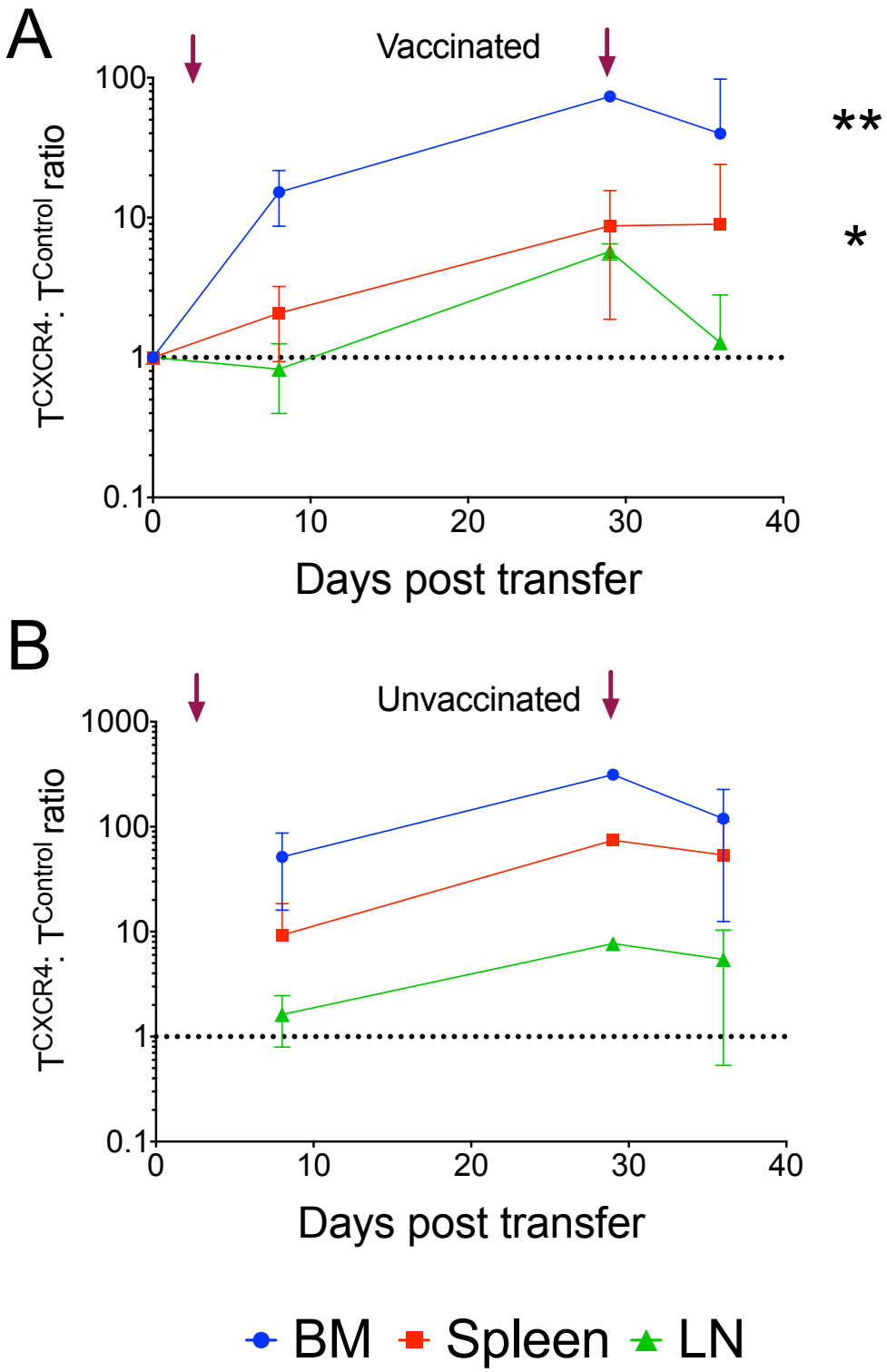


Figure 4-2 Constitutive T^{CXCR4} summary ratio kinetics.
Summary data for (A) $T^{CXCR4:}$ $T^{Control}$ ratio in vaccinated mice (relevant antigen), n=16, data representative of 4 independent experiments, and (B) T^{CXCR4}: $T^{Control}$ ratio in unvaccinated mice (irrelevant antigen), n=8, data representative of 3 independent experiments. Error bars denote SD, *P≤0.05, **P≤0.01 denote significant numerical differences between T^{CXCR4} and $T^{Control}$, purple arrows denote time of peptide injection.

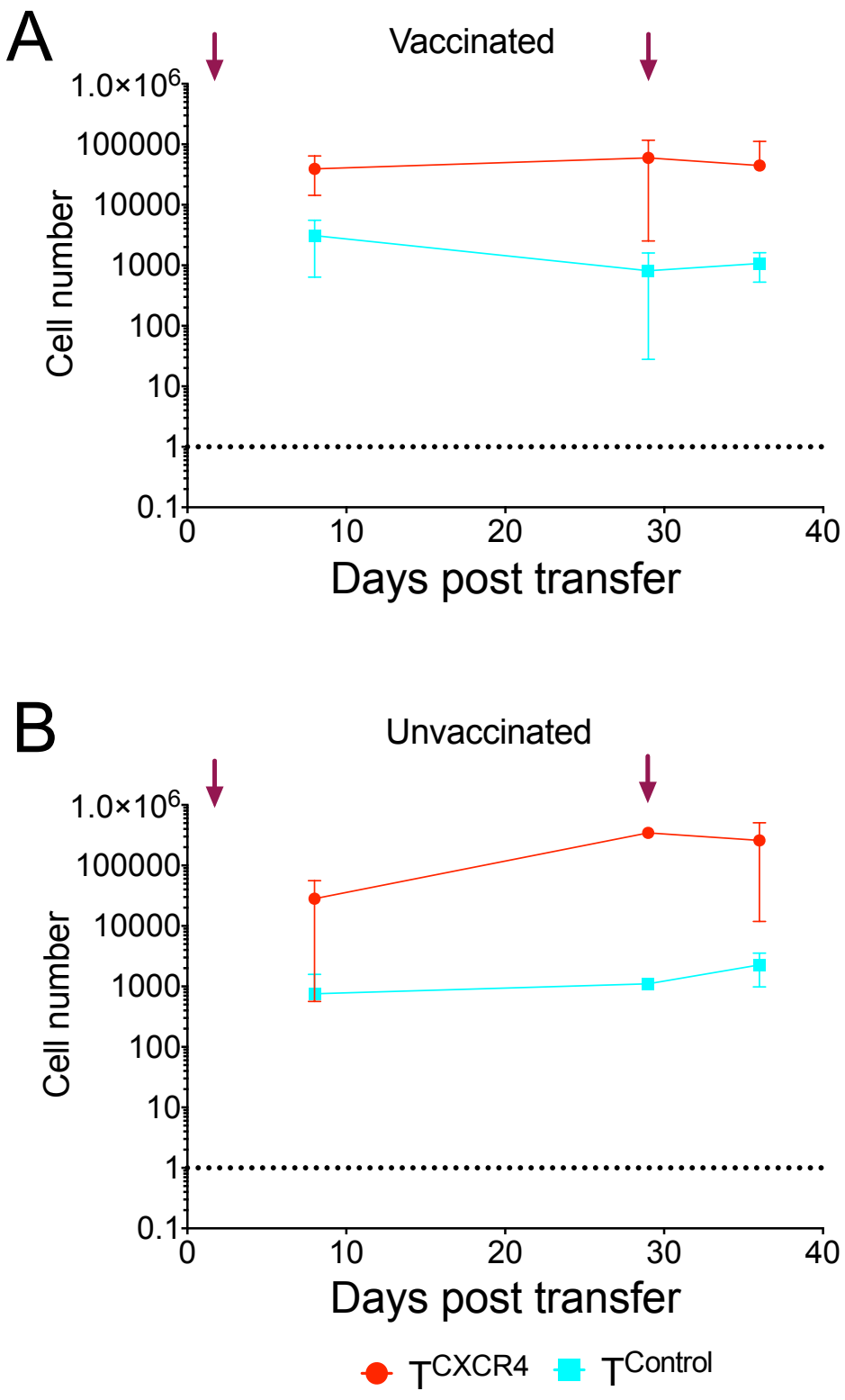


Figure 4-3 Constitutive T^{CXCR4} bone marrow accumulation.
Absolute number data for (A) BM T^{CXCR4} and $T^{Control}$ in vaccinated mice (relevant antigen), n=16, data representative of 4 independent experiments and (B) unvaccinated mice (irrelevant antigen), n=8, data representative of 3 independent experiments. Error bars denote SD, purple arrows denote time of peptide injection.

Memory phenotype

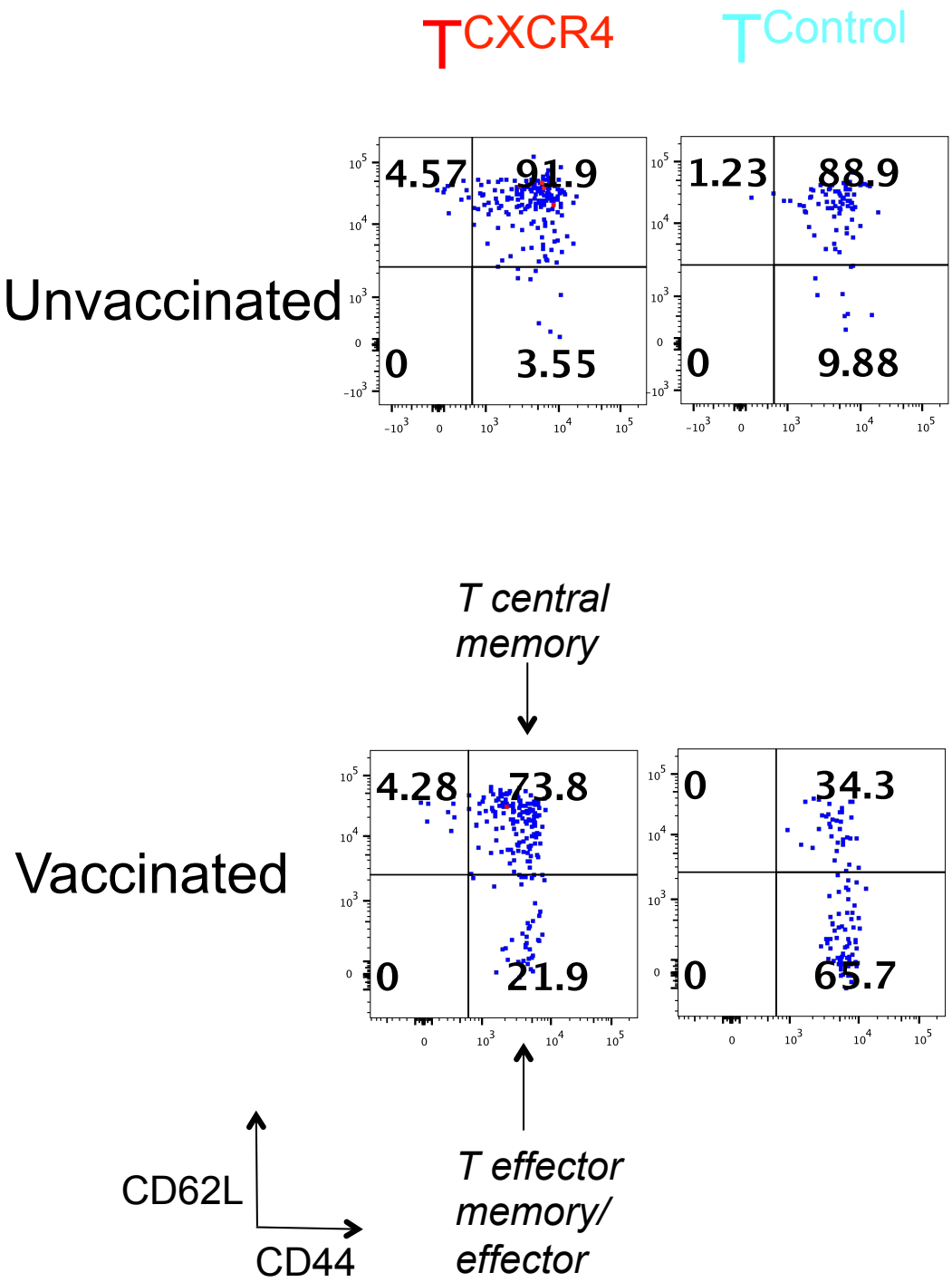
A greater proportion of vaccinated T^{CXCR4} exhibited a central memory (T^{CM}) phenotype in all organs compared to $T^{Control}$ at day 8. The median BM $\%T^{CM}$ was 38% vs 9% (2 tailed paired t-test; p=0.003), spleen T^{CM} 37% vs 13% (p=0.014) and LN 61% vs 38%, p=0.07) (Figure 4-4). In contrast, T^{CM} proportions were similar in unvaccinated mice for all organs (BM 83% vs 89%, spleen 79% vs 77%, LN 87% vs 89%).

Cytokine receptors

To investigate the impact of cytokine signalling in the lymphopenic environment, I stained for the key components of homeostatic cytokine receptors. IL-2/15Rβ (CD122) expression was similar (T^{CXCR4} BM MFI 2429 vs $T^{Control}$ 2281, spleen 1816 vs 1729). IL7Rα (CD127) expression was decreased on T^{CXCR4} compared to $T^{Control}$ in unvaccinated mice BM and spleen (BM MFI 136 vs 520, spleen 151 vs 742, LN 671 vs 608) but no differences were seen in vaccinated mice as $T^{Control}$ downregulate CD127 to a similar extent as T^{CXCR4} in response to antigen (BM 150 vs 133, spleen 118 vs 181, LN 477 vs 453).

IL2Rα (CD25) expression was slightly lower in T^{CXCR4} vs $T^{Control}$ regardless of antigen exposure (vaccinated BM 206 vs 318, spleen 125 vs 278, LN 45 vs 352, unvaccinated BM 258 vs 362, spleen 152 vs 215, LN 15 vs 216), whilst expression of the common γ-chain receptor (CD132, receptor signalling component for IL-2, -4, -7, 15 and 21) was similar in all conditions (vaccinated BM MFI 465 vs 485, spleen 912 vs 663, LN 1579 vs 1205, unvaccinated BM MFI 592 vs 577, spleen 1381 vs 1312, LN 1939 vs 1686).

A


TCXCR4
TControl
Unvaccinated
4.57
91.9
0
3.55
1.23
88.9
0
9.88
T central memory
Vaccinated
4.28
73.8
0
21.9
0
34.3
0
65.7
CD62L
CD44
T effector memory/ effector

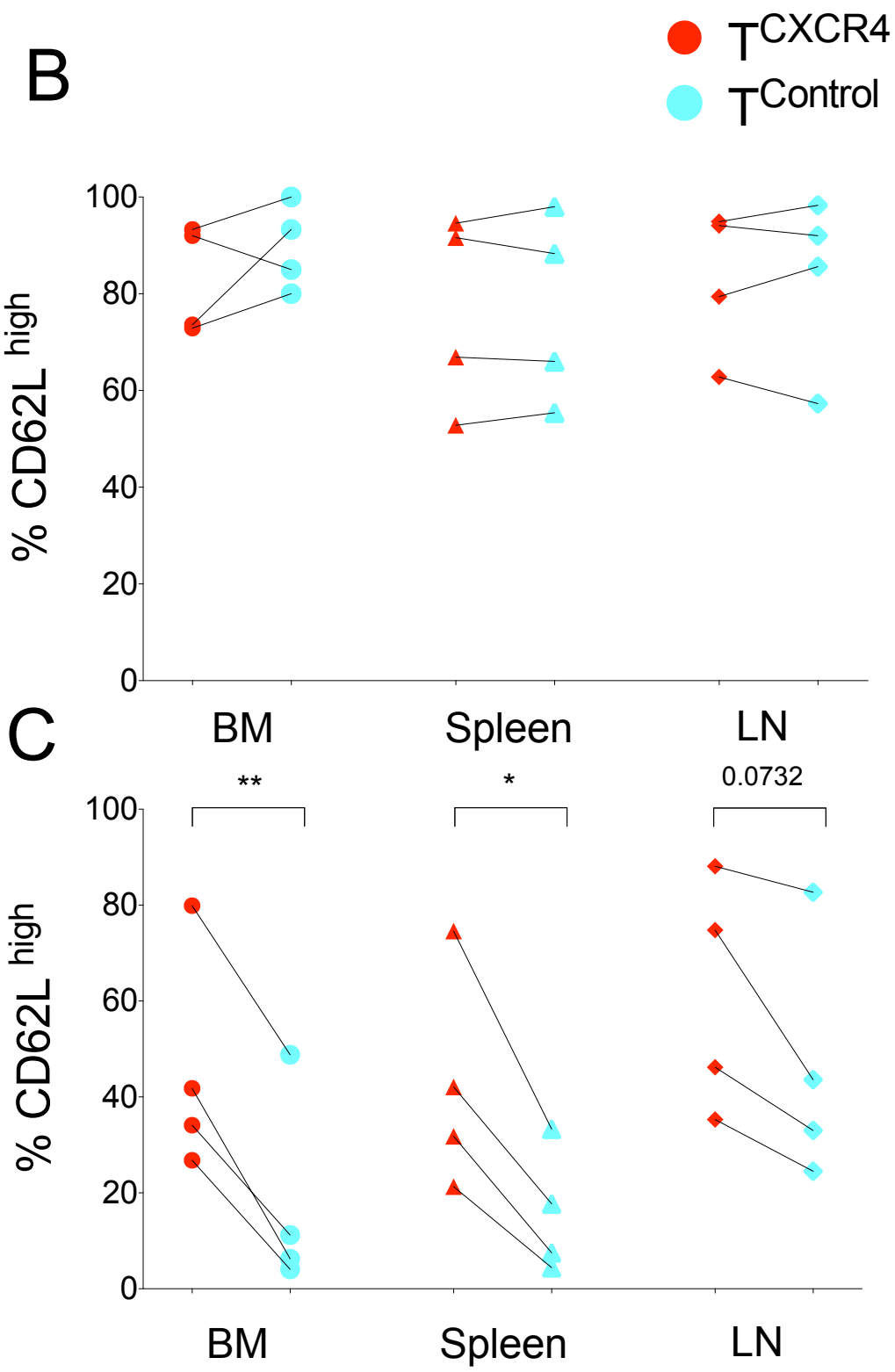


Figure 4-4 T^{CXCR4} retain central memory phenotype post initial vaccination.

(A) Representative day 8 flow cytometry plots depicting CD44/CD62L expression of splenic $CD8^{+}GFP^{+}$ T^{CXCR4} and $T^{Control}$ from the unvaccinated and vaccinated mice. Summary paired day 8 T^{CM}% data for (B) unvaccinated and (C) vaccinated T^{CXCR4} and $T^{Control}$ in lymphoid organs, n=8, data from 3 independent experiments, *P≤0.05, **P≤0.01.

Survival

Next I investigated the expression of survival-related proteins. Expression of pro-survival protein BCL2 was increased in vaccinated mice T^{CXCR4} vs $T^{Control}$ (median % positive in BM 52.3 vs 31.9, spleen 73.2 vs 37.8). BCL2 MFI showed the same pattern (BM 347 vs 163, spleen 583 vs 256).

This difference was not seen in unvaccinated mice (median BM % positive 85.3 vs 82.4, spleen 92.9 vs 90.1). Expression of pro-apoptotic effector caspase 3 was reciprocally decreased in BM and spleen of vaccinated mice for T^{CXCR4} vs $T^{Control}$ (median % positive 0.6 vs 2.7 and 0.5 vs 2.6, MFI 285 vs 328 and 379 vs 446). A similar expression pattern was true for caspase 3 in unvaccinated mice (BM % positive 0.8 vs 4.2 and spleen 2.1 vs 7.3, BM MFI 294 vs 370 and spleen 545 vs 681).

Proliferation

To determine the reason for differences seen in accumulation, I also examined the proliferation rate using 5-ethynyl-2-deoxyuridine (EdU) staining. T^{CXCR4} underwent similar or slightly lower levels of proliferation in both the vaccinated (BM EdU % positive 2.1 vs 11.5 and spleen 5.4 vs 9.6, 2 tailed paired t test; p=0.081 and 0.096 respectively) and unvaccinated setting (BM 1.7 vs 1.0 and spleen 5.0 vs 11.5, p=0.074 and ns). EdU MFI was similar for all groups (BM vaccinated T^{CXCR4} median MFI 3033 vs $T^{Control}$ 3453 and spleen 1651 vs 1920, unvaccinated BM 3522 vs 3563 and spleen 2016 vs 2117, p=ns for all).

Function

Finally, I examined the functional ability of the T cells to produce effector cytokines after ex vivo re-stimulation of splenocytes with relevant peptide. T^{CXCR4} exhibited slightly reduced IFN-γ production for vaccinated mice (median % positive 39.8 vs 53.4) and similar levels in unvaccinated mice (49.3 vs 50.0).

Primary vaccination summary

T^{CXCR4} accumulate in greater numbers than $T^{Control}$ in spleen, dramatically so in bone marrow, and to a heightened extent in the absence of antigen. After antigen encounter, a significantly higher proportion of T^{CXCR4} maintain a central memory phenotype with higher expression of BCL2 but similar cytokine receptor expression, function and proliferation. These phenotypic differences are not seen in unvaccinated mice. These findings suggest that the early T^{CXCR4} numerical advantage relates primarily to improved survival rather than proliferation, and also indicate that the degree of numerical advantage is partially mitigated by heightened proliferation of $T^{Control}$ in the vaccinated setting (Table 2).

4.3 Steady state post-vaccination phenotype

In order to examine how the change in the initial phenotypes over time in the absence of further exposure to antigen, I then studied these outputs in mice four weeks post initial vaccination, prior to boost vaccination.

Number and ratio

There were greater numbers of BM T^{CXCR4} at day 29 in vaccinated mice - median 60130 (median 27017 at day 8) vs BM $T^{Control}$ median 800 at day 29 (median 2319 at day 8). Day 29 BM T^{CXCR4}: $T^{Control}$ ratio was therefore dramatically enhanced at a median of 73:1. The same was true to an even greater degree in unvaccinated mice – BM median absolute number T^{CXCR4} 345451 vs $T^{Control}$ 1103 and ratio 313:1 (Figure 4-2). The same pattern was true to a lesser extent for all other organs. Spleen absolute T^{CXCR4} number was 42,442 at day 29 (20740 at day 8) vs $T^{Control}$ 5,122 (day 8 10355). Spleen T^{CXCR4}: $T^{Control}$ ratio was 8.3:1. There were greater numbers of T^{CXCR4} cells in unvaccinated spleen (361898 vs 4872, ratio 74.3:1). Lymph node T^{CXCR4} showed a similar marked advantage (vaccinated ratio 5.9:1 and unvaccinated 7.7: 1). In summary, these data demonstrate dramatically enhanced persistence of T^{CXCR4} and higher numbers at day 29 compared to day 8 in all organs in vaccinated mice, despite the absence of further antigen. The same was true to an even greater extent for T^{CXCR4} in unvaccinated mice. In contrast, numbers of $T^{Control}$ diminished over time in the vaccinated group, and

failed to increase significantly in the unvaccinated group. In particular very few $T^{Control}$ cells remained in unvaccinated lymph node and bone marrow.

Memory phenotype

A higher proportion of T^{CXCR4} maintained a central memory phenotype at Day 29 in all organs in vaccinated mice (BM 81.1% vs 14.8%, spleen 79.2% vs 22.9%, LN 96.3% vs 56.9%) (Figure 4-5). A similar effect of limited magnitude was seen in mice vaccinated with non-cognate peptide (BM 95.1% vs 79.4%, spleen 94.6% vs 81.6%, LN 96.2% vs 92.1%).

Cytokine receptors

After prolonged exposure to the lymphopenic environment at day 29, vaccinated T^{CXCR4} expressed significantly higher levels of IL-15Rβ (CD122) compared to $T^{Control}$ in BM (MFI 1170 vs 437, 2 tailed paired t-test; $p<0.001$) and spleen (MFI 925 vs 438; $p=0.005$) and similar levels in lymph node (MFI 1165 vs 974, p=ns). Common γ-chain receptor (CD132) expression was also significantly higher in vaccinated BM (599 vs 471, $p=0.05$), and similar in spleen (920 vs 559) and LN (1327 vs 1114). IL7Rα (CD127) was similar in all organs (BM MFI 494 vs 431, spleen 502 vs 445, LN 837 vs 1218, p=ns). CD25 was not significantly different between T^{CXCR4} and $T^{Control}$ in all organs (BM 0 vs 13, spleen 0 vs 103, LN 3 vs 113, p=ns).

Survival and Proliferation

At day 29, T^{CXCR4} showed a similar pattern of significantly higher BCL2 expression in T^{CXCR4} vs $T^{Control}$ (median BM % positive 46.8 vs 25.0, spleen 43.6 vs 18.1, 2 tailed paired t-test; p=0.03 and 0.04 respectively). Caspase expression was not significantly different (BM MFI 325 vs 313, spleen 430 vs 393, p=0.55 and 0.07). EdU proliferation was equivalent for T^{CXCR4} vs $T^{Control}$ (BM MFI 1172 vs 1117 and spleen 920 vs 800, p=ns for both).

Function

Upon ex-vivo restimulation of splenocytes with cognate peptide, previously vaccinated T^{CXCR4} displayed superior cytokine responses to $T^{Control}$ in terms of IFN-γ (median % positive 42.3 vs 33.3 and MFI 401 vs 205, 2 tailed paired t-test; p=0.08 and 0.02), TNF-α (median % positive 37.5 vs 14.5 and MFI 1657 vs 562, p=0.04 and 0.20) and IL-2 (median % positive 14.6 vs 2.5 and 410 vs 109, p=0.25 and 0.03).

Steady state phenotype summary

The numerical advantage of T^{CXCR4} was maintained and increased over time in all organs regardless of prior vaccination. In vaccinated mice, the frequencies of T^{CXCR4} maintaining a central memory phenotype were dramatically higher than $T^{Control}$, and also higher than T^{CXCR4} post primary vaccination. Vaccinated BM and splenic T^{CXCR4} expressed higher levels of stem memory phenotype markers IL-15Rβ and BCL2 and demonstrated

greater functional responses upon ex vivo restimulation. The prolonged period prior to secondary vaccination was designed to identify phenotypic changes and ability to access homeostatic cytokine, and indicated that T^{CXCR4} possess the ability to maintain a less differentiated central memory phenotype regardless of initial antigen exposure.

4.4 Recall response following boost vaccination

I then re-vaccinated mice four weeks post primary vaccination at day 29 and determined phenotypes one week post boost at day 36 to establish the effects of repeated antigen exposure.

Number and ratio

There were significantly increased absolute numbers of vaccinated T^{CXCR4} compared to $T^{Control}$ in spleen and BM at day 36. Median BM numbers were 12,477 vs 1087 (Wilcoxon signed rank; p=0.004) (Figure 4-3), spleen 19,170 vs 7620 (p=0.027) and lymph node 482 vs 1323 (p=ns). Median T^{CXCR4}: $T^{Control}$ ratios were also significantly higher in BM (14.1:1, Wilcoxon signed rank; p=0.004) (Figure and spleen (2.2:1, p=0.02) and similar in lymph node (0.7:1, p=ns), (Figure 4-2). These differences were magnified in unvaccinated mice for BM and spleen, but lacked significance due to a replicate value of 3. Absolute number was 264,450 for unvaccinated T^{CXCR4} BM vs $T^{Control}$ 1875,

and spleen T^{CXCR4} were 140,282 vs 3129, LN 1098 vs 874. Unvaccinated ratios were similarly increased (BM 136:1, spleen 45.8:1, lymph node 5.3:1).

Memory phenotype

The proportion of central memory cells was significantly greater post boost vaccination in all organs, BM median T^{CM}% was 71.3 vs 23.7, spleen 50.5% vs 16%, lymph node 66.4% vs 37.7%, Wilcoxon's signed rank; p=0.004 for all) (Figure 4-5). This difference was not present in unvaccinated T^{CXCR4} and $T^{Control}$ where T^{CM} proportions were similar to each other. Thus, over time T^{CXCR4} retain higher levels of CD62L in all organs despite repeated antigenic challenge (Figure 4-5).

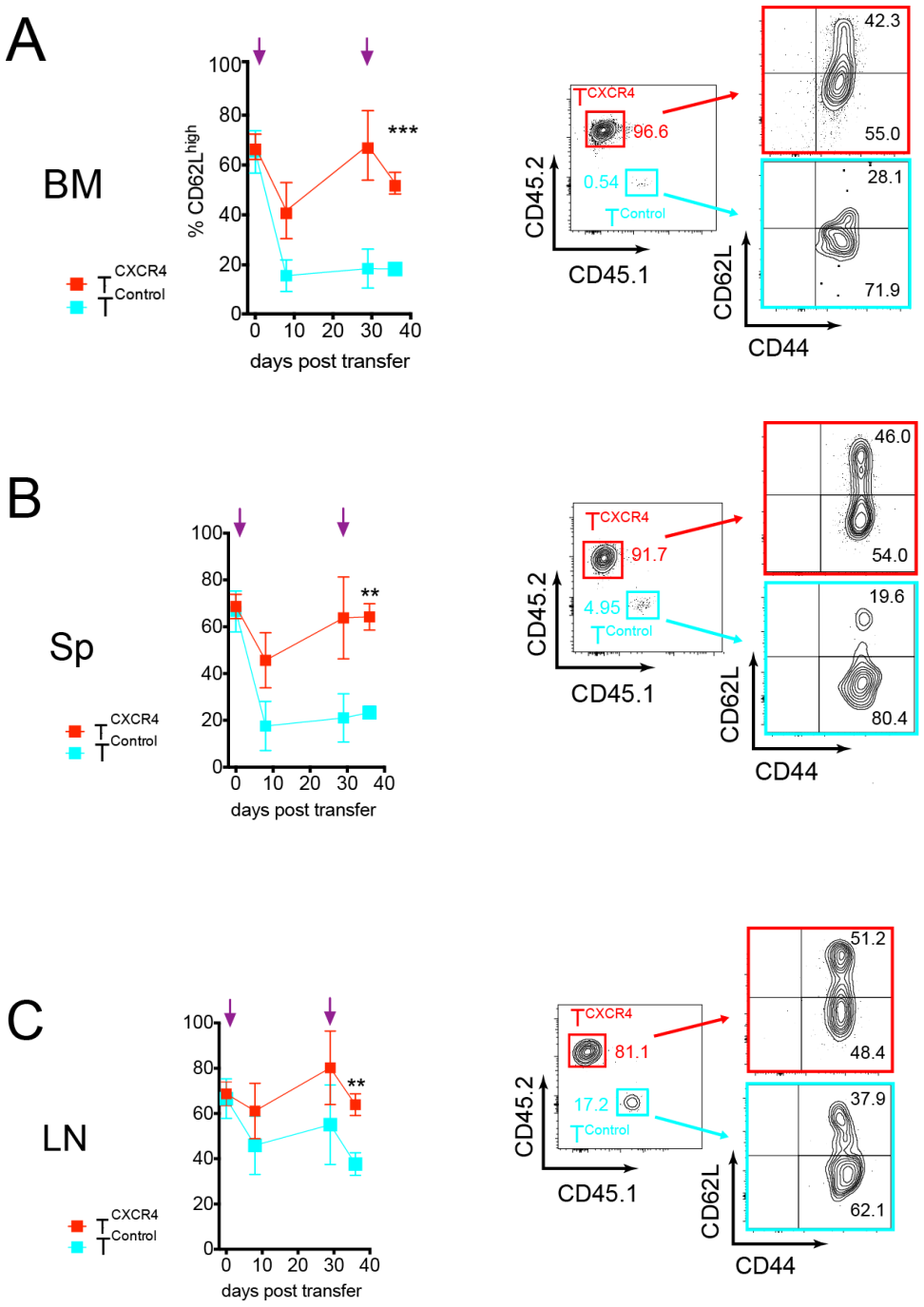


Figure 4-5 T^{CXCR4} retain a central memory phenotype after repeated antigen exposure

%$CD62L^{high}$ T^{CXCR4} (red) and $T^{Control}$ (cyan) over time after SIINFEKL peptide vaccination (purple arrows) at days 1 and 29 following transfer into *Rag1ko* mice and representative flow plots denoting relative proportions of $CD8^{+}GFP^{+}$ T cells and day 36 surface expression of CD44/CD62L. (A) BM, (B) Spleen and (C) LN were harvested at days 8, 29 and 36 post transfer. Error bars denote SD, **P<0.01; ***P<0.001, n=16, data representative of 4 independent experiments.

Importantly, although the proportion of CD62L^{low} T$^{EM/EFF}$ cells was lower for OT-I T^{CXCR4} vs T^{Control}, their absolute number was higher in BM and equivalent in other organs (Figure 4-6) suggesting that greater expansion of the entire T^{CXCR4} population enables superior production of both memory and effector CD8^{+} T cells.

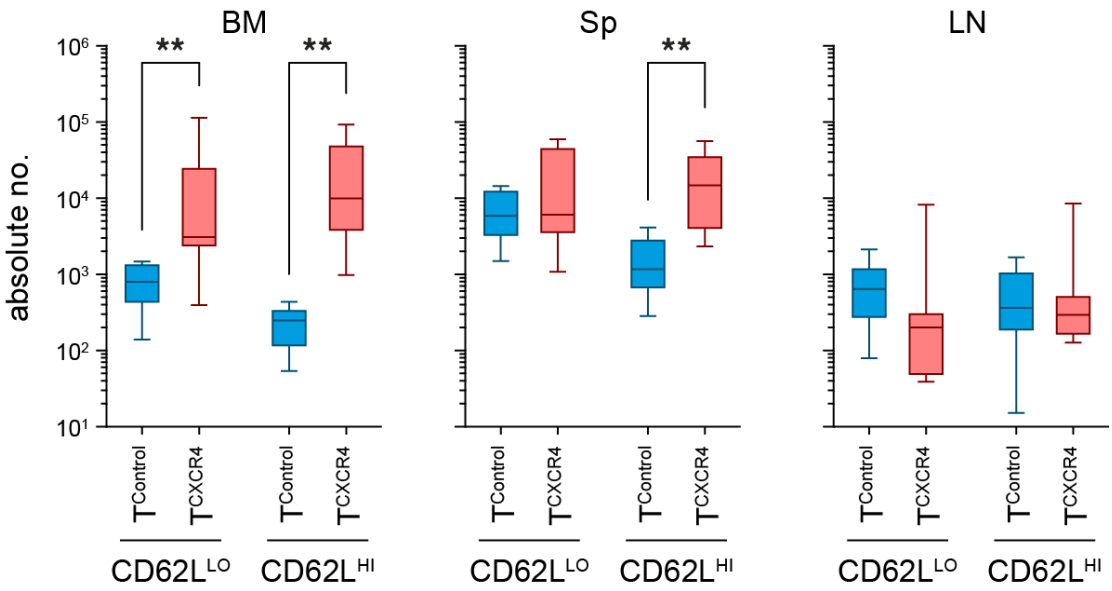


Figure 4-6 Absolute numbers of Ag-activated CD62L^{high} and CD62L^{low} transduced CD8^{+} T cells

Box and whisker plots depicting day 36 numbers of TCXCR4 (red) and TControl (cyan) post vaccination, n=9, data representative of 4 independent experiments, **P<0.01.

Cytokine receptors

Post boost vaccination, T^{CXCR4} expressed significantly higher levels of CD122 (IL-15Rβ) compared to T^{Control} in bone marrow (MFI 2408 vs 1939, Wilcoxon's signed rank; p=0.008) (Figure 4-7) and spleen (2048 vs 1798, p=0.020) and similar levels in LN (MFI 2328 vs 2279, p=ns). In contrast, values were similar

for all organs in unvaccinated mice (BM 1624 vs 1728, spleen 1594 vs 1658, LN 1594 vs 1544).

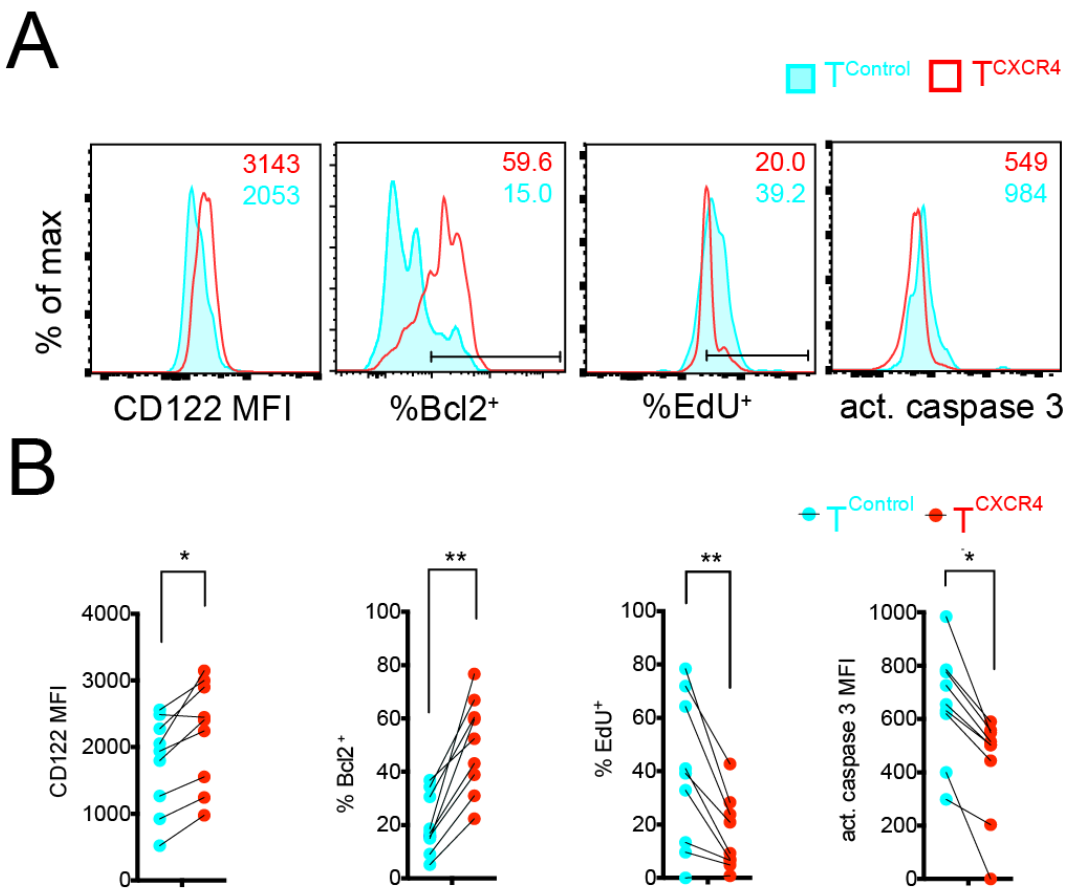


Figure 4-7 Post-boost vaccination memory phenotype.
(A) Representative flow cytometric histograms and (B) summary data for day 36 IL-15Rβ (CD122), BCL2, EdU and activated caspase 3 expression on BM $CD8^+GFP^+$ T^{CXCR4} and $T^{Control}$ post vaccination. Top right figures denote MFI, n=9, data representative of 4 independent experiments. *P<0.05, **P<0.01.

CD127 (IL7Rα) levels were similar in vaccinated mice (BM MFI 326 vs 185, spleen 257 vs 190, LN 549 vs 606, p=ns for all) and CD132 (common gamma chain receptor) levels were significantly lower in BM (MFI 1093 vs 1315, Wilcoxon's signed rank; p=0.012), trended lower for spleen (MFI 1121 vs

1495, p=0.055) and were similar in LN (MFI 1913 vs 1429, p=ns). Finally, CD25 expression was slightly lower in all organs for T^{CXCR4} (BM MFI 0 vs 140, Wilcoxon's signed rank; p=0.039, spleen MFI 0 vs 130, LN MFI 0 vs 220, p=0.004 for both). All cytokine receptor levels remained similar in unvaccinated mice (CD122, CD132, CD127, CD25), demonstrating the requirement for antigen exposure to produce the T^{CXCR4} phenotype.

Survival

The proportion of T^{CXCR4} cells expressing BCL2 was again significantly higher than $T^{Control}$ in all vaccinated organs. Median BCL2 %positive for T^{CXCR4} vs $T^{Control}$ was 52.4% vs 17.0% in BM (Figure 4-7), 37.5% vs 19.4% in spleen and 62.9% vs 47.6% in LN (Wilcoxon's signed rank; p=0.004, 0.004 and 0.008 respectively). The same was true for BCL2 expression (BM MFI 1064 vs 689, spleen 978 vs 633, LN 1598 vs 1098, Wilcoxon's signed rank; p=0.004, 0.004 and 0.008 respectively).

Similarly, caspase 3 positivity was significantly decreased in all vaccinated organs for T^{CXCR4} (BM 1.3% vs 7.5%, spleen 1.5% vs 13.1%, LN 4.1% vs 14.5%, Wilcoxon's signed rank; p=0.027, 0.008 and 0.008 respectively, BM MFI 504 vs 655, spleen 640 vs 805, LN 835 vs 887, Wilcoxon's signed rank; p=0.004, 0.008 and ns respectively (Figure 4-7). No differences in BCL2 or caspase 3 were seen in unvaccinated mice.

Proliferation

Vaccinated BM T^{CXCR4} proliferated to a slightly lower extent compared to $T^{Control}$. BM EdU MFI was 2812 vs 3609 (Wilcoxon's signed rank; p=0.02) (Figure 4-7), spleen 2311 vs 2609 (p=ns) and LN 1987 vs 2043 (p=ns). Levels of proliferation were similar in the unvaccinated setting (BM MFI 1609 vs 1588, spleen MFI 1152 vs 1125, p=ns for both).

Function

Upon ex vivo splenocyte restimulation, vaccinated T^{CXCR4} alone expressed significantly higher levels of all measured cytokines compared to $T^{Control}$. Median IFN-γ-% positive was 50.8% vs 27.5%, TNF-α % positive 25.8% vs 9.8% and IL-2 % positive 5.6% vs 1.3% (Wilcoxon's signed rank; p=0.016, 0.008 and 0.008 respectively) (Figure 4-8A and B). The same was true for MFI: IFN-γ 545 vs 135, TNF-α 642 vs 521 and IL-2 MFI 253 vs 196 (p=0.016, 0.008 and 0.027). I applied Boolean gating to measure the frequency of polyfunctional cells (cells expressing 2 or more cytokines). These were significantly increased for T^{CXCR4} (median 22.4% vs $T^{Control}$ 8.0%, p<0.001), as were all cytokine positive combinations (IFN-γ and TNF-α, IFN-γ and IL-2, TNF-α and IL-2) and trifunctional cells secreting all 3 cytokines (2.2% vs 0.7%, p=0.02) (Figure 4-8C). These differences were not seen in unvaccinated mice.

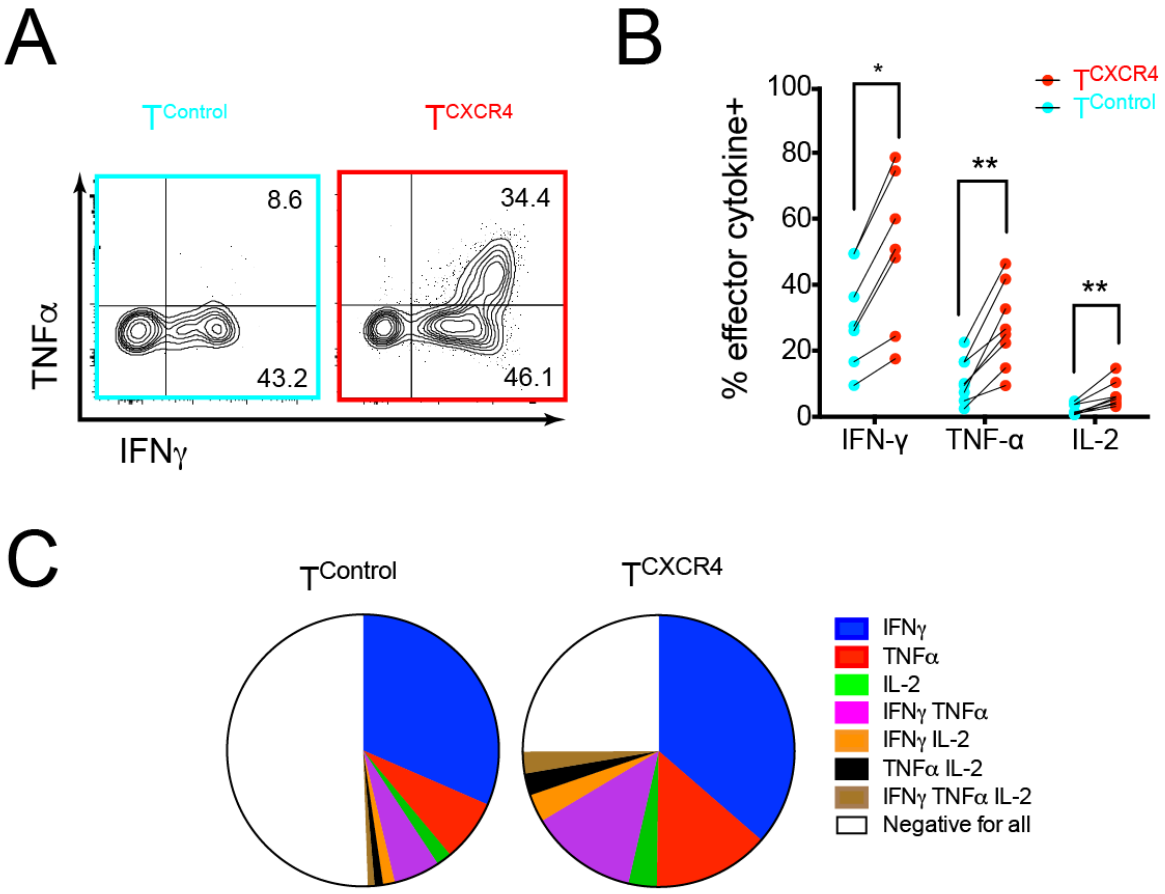


Figure 4-8 Cytokine production and polyfunctionality.
(A) Representative flow cytometric plots denoting intracellular IFN-γ and TNF-α co-staining by T^{CXCR4} and $T^{Control}$ at 4hrs post ex vivo SIINFEKL stimulation. Gating performed on vaccinated $CD8^{+}GFP^{+}$ splenocytes at day 36. (B) Summary data for %IFN-γ^{+}, TNF-α^{+} and IL-2^{+} production from vaccinated day 36 T^{CXCR4} and $T^{Control}$ splenocytes, 4 hours post ex vivo SIINFEKL stimulation, n=7, data representative of 4 independent experiments. (C) Pie charts depicting polyfunctional cytokine generation summary data according to Boolean combination gates identifying IFN-γ^{+}, TNF-α^{+} and IL-2^{+} cells. *P<0.05; **P<0.01.

Senescence and exhaustion

In order to investigate the effects of repeated antigen exposure upon vaccinated cells, I also investigated expression of the T-cell exhaustion and senescence markers KLRG1 and PD-1, and relevant transcription factors (T-bet and Eomes). I used splenic samples because of insufficient BM $T^{Control}$ for comparison. Splenic T^{CXCR4} demonstrated significantly lower levels of PD-1 % positivity than $T^{Control}$ (median 18.8% vs 60.5%, Wilcoxon's signed rank; p=0.031) and lower PD-1 MFI (183 vs 495, p=0.031). In addition, splenic T^{CXCR4} expressed lower levels of KLRG1 (MFI 44.8 vs 500, p=0.031). Splenic T^{CXCR4} were also characterised by significantly higher frequencies of a memory-associated $Eomes^{High}$ T-bet^{Low} phenotype (median 52% vs 44%, Wilcoxon's signed rank; p=0.031). Recent data has highlighted important differences between PD-1 expressing cells, differentiating between PD-1^{mid} T-bet^{high} cells which can be reinvigorated by antibody blockade, and terminally differentiated, 'hyper-exhausted' PD-1^{high} $Eomes^{high}$ cells which cannot (359, 488). The proportion of hyper-exhausted splenic $T^{Control}$ was markedly greater than T^{CXCR4} (median 65.8% vs 34.0%, p=0.015) (Figure 4-9).

In an alternative conceptual model, antigen-specific T cells can be subdivided into memory precursor (MPECs) and short-lived effector cells (SLECs) according to KLRG1 and CD127 expression. When analysed according to this framework, T^{CXCR4} maintain significantly higher proportions of MPECs and lower numbers of SLECs in the spleen post boost vaccination (MPECs 35.8% vs 10.9% and SLECs 7.9% vs 40.2%, Wilcoxon's signed rank; p=0.031 for both), (Figure 4-9).

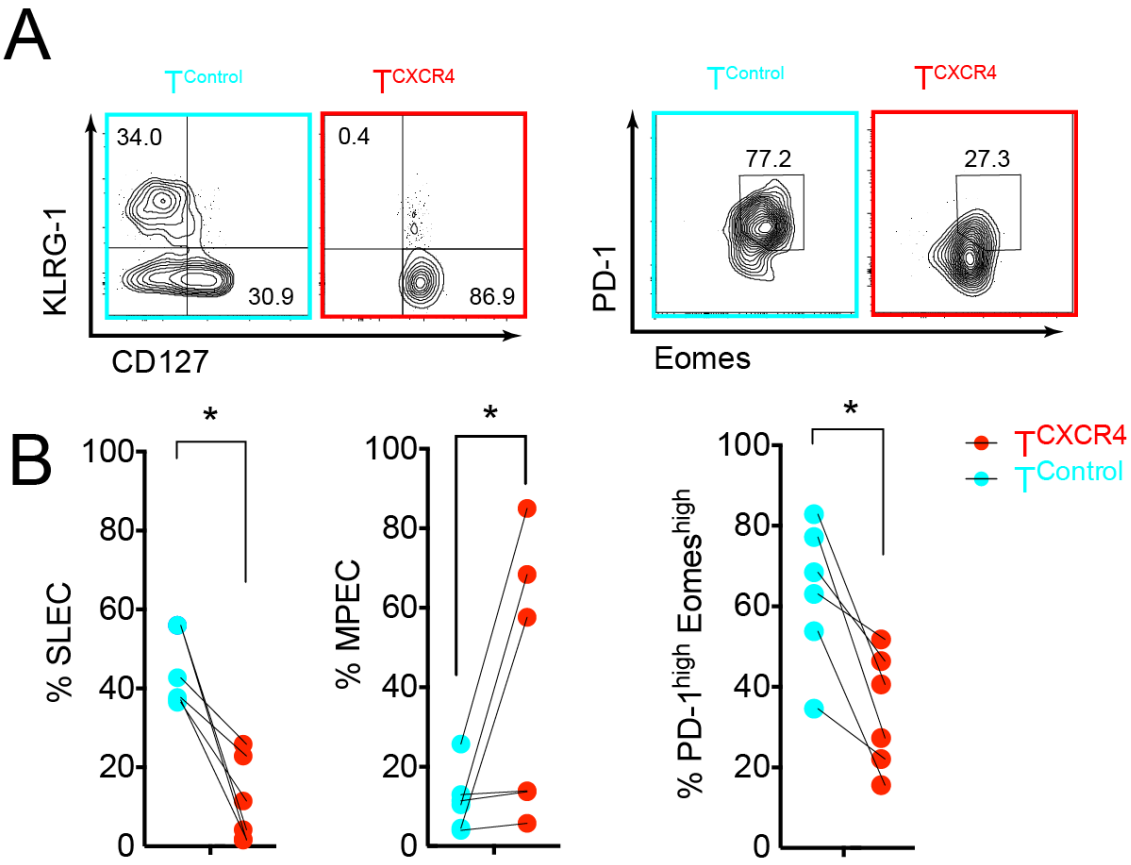


Figure 4-9 Exhaustion and senescence phenotype.
(A) Exemplar flow plots and (B) summary data showing frequency of day 36 splenic T^{CXCR4} and $T^{Control}$ SLECs ($KLRG1^{high}$ $CD127^{low}$), MPECs ($KLRG1^{low}$ $CD127^{high}$) and hyper-exhausted phenotypes ($PD\text{-}1^{high}$ $Eomes^{high}$), n=6, data representative of 1 independent experiment, *P<0.05.

Boost vaccination summary

T^{CXCR4} maintain a dramatically increased numerical advantage compared to $T^{Control}$ in bone marrow and spleen after boost vaccination. This numerical advantage is also enhanced in all organs in the absence of antigen, suggesting superior ability to access homeostatic cytokine. After antigen exposure, T^{CXCR4} maintain markedly higher proportions of T^{CM}, IL-15Rβ and BCL2 expression in all organs. T^{CXCR4} also exhibit higher levels of effector cytokine expression upon restimulation, and higher frequencies of

polyfunctionality. Finally, consistent with an early memory phenotype, T^{CXCR4} are characterised by higher frequencies of a memory-associated $Eomes^{High}$ T-bet^{Low} transcription factor profile and express very low levels of the inhibitory exhaustion marker PD-1 and terminal senescence marker KLRG1 despite repeated antigenic stimulation. These early memory phenotypes, summarised in Table 2 below, are not seen in the absence of antigen exposure.

Phenotype	Interpretation	T^{CXCR4}	$T^{Control}$	Significance
BM accumulation	↑	12477 (1353-195694)	1087 (189-1803)	P=0.004
BM %T^{CM}	↑	71.3 (37.9-82.8)	23.7 (10.1-33.0)	P=0.004
BM IL-15Rβ expression (MFI)	↑	2408 (978-3145)	1939 (521-2558)	P=0.008
BM IL-7Rα expression (MFI)	-	326 (138-576)	185 (121-707)	P=ns
BM $BCL2\%^{+}$	↑	52.4 (22.4-76.7)	17.0 (5.1-36.8)	P=0.004
BM caspase $3\%^{+}$	↓	1.3 (0.0-2.5)	7.5 (0.0-21.2)	P=0.027
EdU MFI	↓	2812 (132-4643)	3609 (0-6091)	P=0.020
IFN-γ$\%^{+}$	↑	50.8 (17.6-78.6)	27.5 (9.5-49.5)	P=0.016
TNF-α%+	↑	25.8 (9.5-46.4)	9.8 (2.5-22.6)	P=0.008
Polyfunctional %	↑	22.4 (13.6-29.3)	8.0 (3.3-18.0)	P<0.001
% MPEC	↑	35.8 (5.7-85.0)	10.9 (4.0-25.7)	P=0.031
% SLEC	↓	7.9 (1.6-25.9)	40.2 (36.5-56.0)	P=0.031
% Hyper-exhausted ($PD\text{-}1^{high}$ $Eomes^{high}$)	↓	34.0 (15.6-51.8)	65.8 (34.6-82.9)	P=0.015

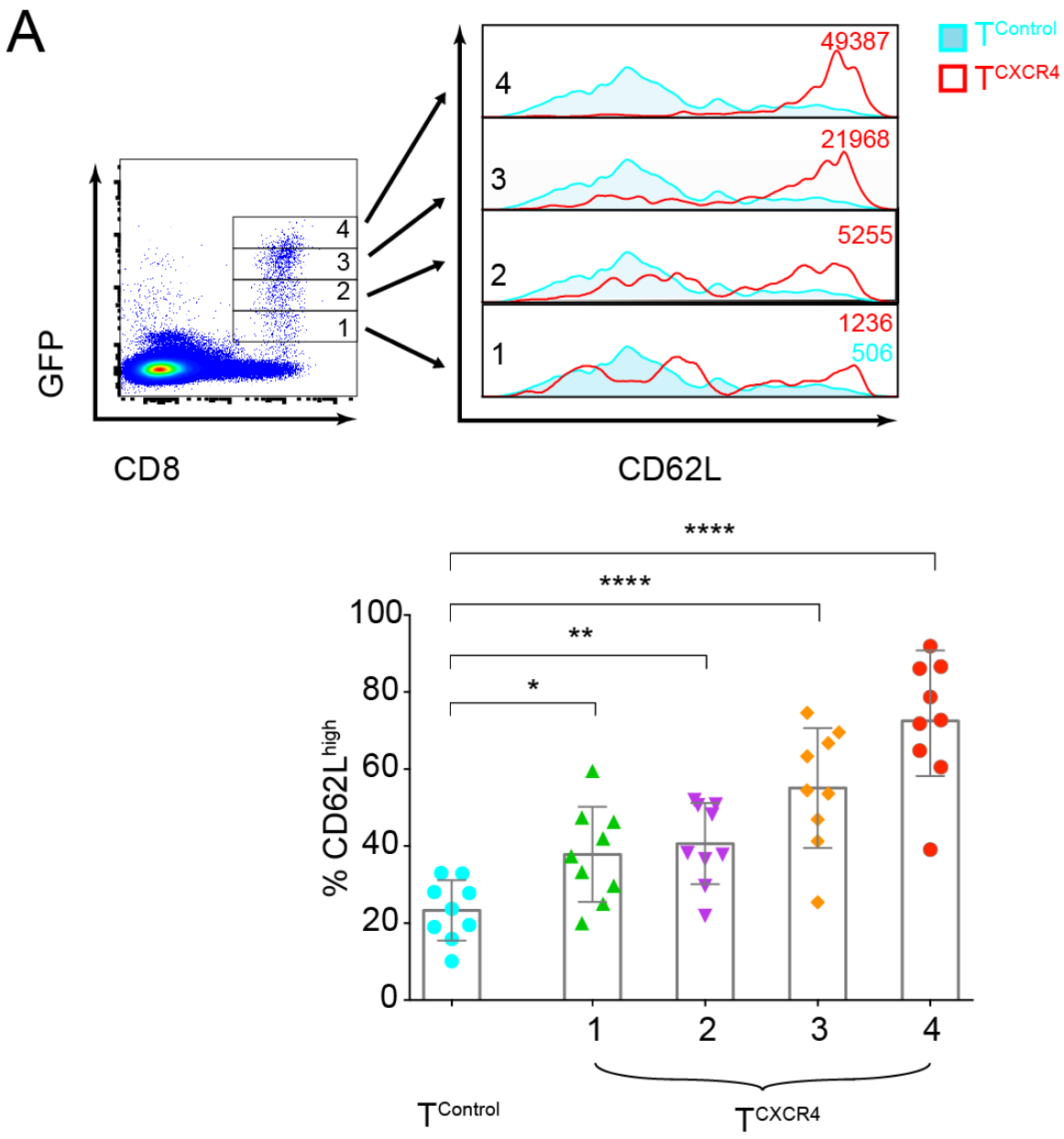
Table 2 T^{CXCR4} recall phenotype post boost vaccination.
Day 36 summary data from SIINFEKL-vaccinated mice. Numbers indicate median (95% confidence intervals). ↑ denotes significantly increased values for T^{CXCR4} relative to $T^{Control}$, ↓ denotes reduced for T^{CXCR4} relative to $T^{Control}$ and - denotes statistical equivalence. Cytokine production, polyfunctionality, %MPEC, SLEC and % hyper-exhausted data from splenic T cells. All p-values Wilcoxon's signed rank test.

4.4 Graded CXCR4 expression modulates the T^{CXCR4} phenotype

I next examined the importance of the level of CXCR4 expression upon the phenotype generated. The transduced cells express a range of CXCR4 correlating with GFP expression (Figure 3-2). Based upon the phenotype seen, I reasoned that $CXCR4^{High}$ T^{CXCR4} would be skewed towards the central memory phenotype.

I therefore compared the change in phenotype for cells according to an increasing gradient of GFP expression. When GFP positive cells are subdivided according to degree of CXCR4 expression (GFP groups 1-4 where 1 denotes GFP MFI 100-400 and 4 denotes the highest level of expression at GFP MFI >5000), an increased proportion of T^{CM} was seen as CXCR4 expression increased in all organs. No change was seen in T^{CM} frequency as GFP MFI increased in $T^{Control}$.

Median BM T^{CM} frequencies were 23.7% $T^{Control}$ vs 37.4% T^{CXCR4} group 1 (2 tailed paired t-test p=0.012), 38.2% for group 2 (p=ns vs T^{CXCR4} group 1), 54.5% for group 3 (p=0.002 vs group 2) and 72.7% for group 4 (p<0.001 vs group3) (Figure 4-10). Similarly splenic T^{CM} frequencies were 16.0% $T^{Control}$ vs


A
GFP
CD8
CD62L
4
3
2
1
49387
21968
5255
1236
506
TControl
TCXCR4
% CD62Lhigh
100
80
60
40
20
0
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TControl
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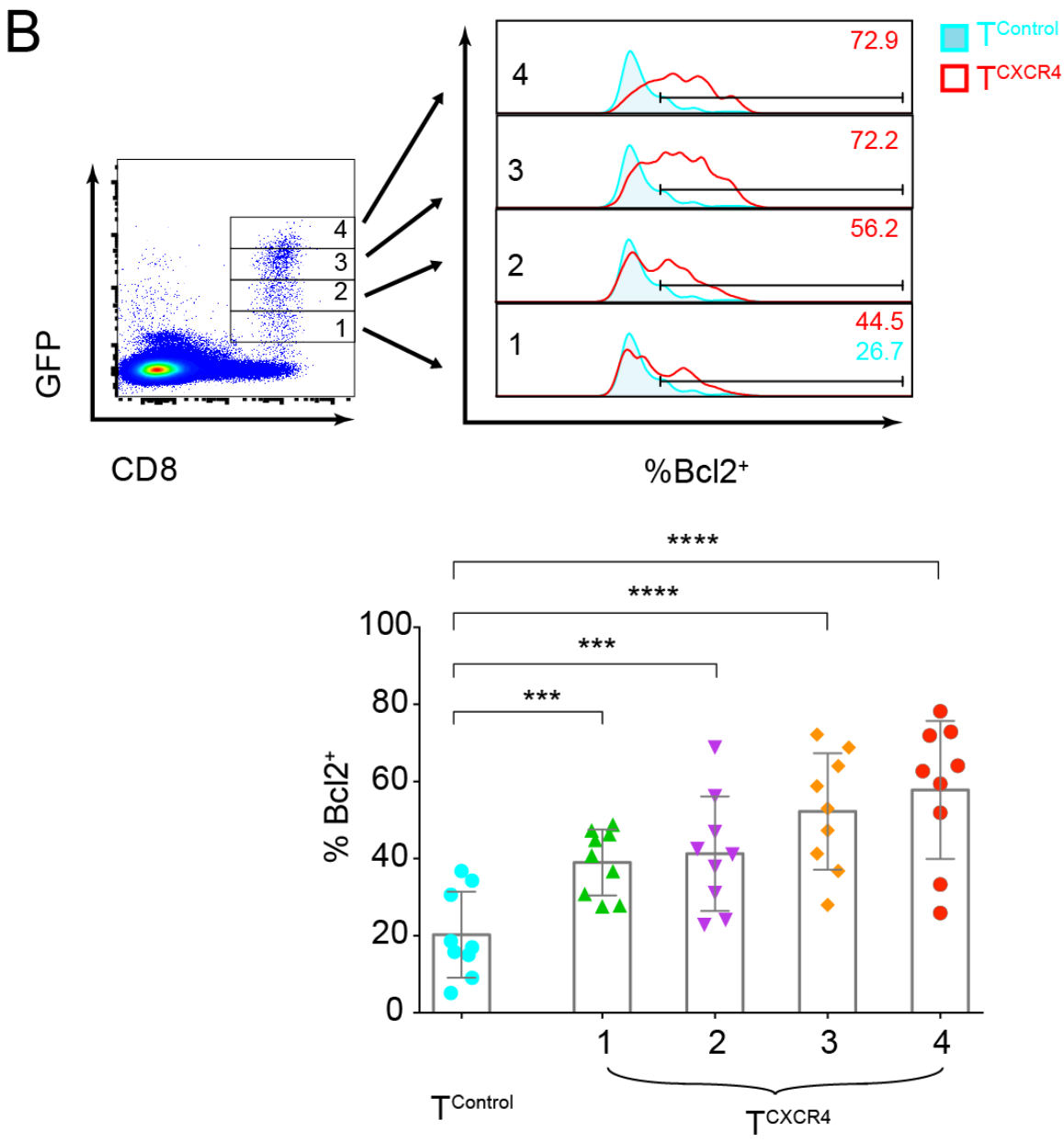

Figure 4-10 T^{CXCR4} memory phenotype correlates with level of CXCR4 expression.

(A) Representative flow plots showing GFP as a marker for CXCR4 expression in vaccinated BM OT-I T^{CXCR4} at day 36, divided into 4 gates (1-4) denoting increasing CXCR4 expression. Histograms show CD62L expression within each gate for BM OT-I $T^{Control}$ (cyan) and T^{CXCR4} (red) and bar graphs show summary data. Numbers top right denote CD62L MFI. (B) As above for BCL2 expression within each gate. Numbers top right denote % BCL2 positive. Error bars denote SD, n=9, data representative of 4 independent experiments *P≤0.05; **P≤0.01; ***P≤0.001, ****P≤0.0001, ns=not significant.

23.2 T^{CXCR4} group 1 (p=0.013), 28.5% group 2 (p=0.002 vs group 1), 44.2% group 3 (p=0.016 vs group 2) and 64.4% group 4 (p=0.006 vs group 3).

The same differences were shown when T^{CM} frequencies were compared in matched GFP groups although there were few $T^{Control}$ cells expressing the highest levels of GFP (median BM T^{CM} frequency in $T^{Control}$ was 20% for group 1, 22.9% group 2, 20.7% for group 3 and 0% for group 4 (2 tailed paired t-test for comparison with matched T^{CXCR4}; p=0.022, 0.001, 0.001 and <0.0001 respectively). Similarly splenic T^{CM} frequency in $T^{Control}$ was 15.8% for group 1, 14.8% group 2, 15.6% group 3 and 7.9% for group 4 (p=0.013, 0.006, <0.0001 and <0.0001 respectively).

The pattern was less pronounced in terms of BCL2 expression, suggesting that a threshold level of BCL2 may be required for this phenotype. BM $T^{Control}$ were 17.0% median BCL2 positive vs 40.8% T^{CXCR4} group 1 (p<0.001), 41.1% group 2 (p=ns vs group 1), 53.0% group 3 (p=0.001 vs group 2) and 62.7% group 4 (p=0.08 vs group 3). Similar findings were shown for the BM homing advantage, with no increase in the homing advantage above a set level of GFP expression (T^{CXCR4}: $T^{Control}$ ratio 0.7:1 for group 1, 3.4:1 for group 2 (Wilcoxon matched-pairs signed rank p=0.004 vs group 1), 6.7:1 for group 3 (p=0.004 vs group 2) and 5.2:1 for group 4 (p=ns vs group 3), (Figure 4-10).

4.5 T^{CXCR4} central memory phenotype and BM accumulation are IL-15 dependent

Data in Figure 3-1 demonstrated a requirement for IL-15 signalling to mediate superior BM accumulation of polyclonal T^{CXCR4}. I hypothesised that the absence of IL-15 signalling would affect BM accumulation and central memory phenotype of T^{CXCR4} in the context of exposure to antigen. I therefore conducted vaccination experiments using OT-I CD8+ T cells transduced with CXCR4-GFP or CTRL-GFP in 1:1 competition experiments, injected into either *Rag1ko* or *IL-15Rako* mice.

Number and ratio

The BM homing advantage of T^{CXCR4} over $T^{Control}$ seen in *Rag1ko* mice after boost vaccination was not seen in *IL-15Rako* mice (Figure 4-11A) (T^{CXCR4}: $T^{Control}$ ratio 2.3:1, Wilcoxon signed rank; p=ns). The ratio of T^{CXCR4}: $T^{Control}$ was also further diminished in spleen and LN (ratio 0.5:1 & 0.2:1, p=ns for both).

Memory phenotype

The preferential maintenance of a central memory phenotype was also reduced in the *IL-15Rako* setting. In BM at day 36, T^{CM} frequencies were only 24.2% vs 8.8% in T^{CXCR4} vs $T^{Control}$ (Wilcoxon's signed rank; p=0.031 (Figure

4-11B) compared with 71.3% for T^{CXCR4} in the *Rag1ko* setting. Spleen and LN-derived T^{CXCR4} no longer demonstrated a significant difference from control cells (Figure 4-11C and D). Splenic T^{CM}% was reduced at 24.7% T^{CXCR4} vs 6.6% $T^{Control}$ (p=ns), down from 50.5% in the T^{CXCR4} *Rag1ko* setting. LN T^{CM}% was 38.9% vs 24.6% (p=ns), down from 66.4% for T^{CXCR4} in *Rag1ko* hosts.

Cytokine receptors

The enhancement in IL-15Rβ expression seen for T^{CXCR4} in vaccinated *Rag1ko* mice was also diminished in *IL-15Rako* hosts. CD122 expression was now only slightly higher for T^{CXCR4} over $T^{Control}$ at day 36 in BM (MFI 2031 vs 1775, Wilcoxon's signed rank; p=0.03 (Figure 4-12A) compared to 2408 in *Rag1ko* mice, and equivalent in spleens and LNs (MFI 1471 vs 1386 and 1624 vs 1732 respectively, p=ns for both).

BM CD132 expression remained slightly lower for T^{CXCR4} in *IL-15Rako* mice (MFI 1112 vs 1355, Wilcoxon's signed rank; p=0.031) and similar for spleen and LN (1046 vs 1079 and 770 vs 1049, p=ns for both). CD127 was similar between T^{CXCR4} and $T^{Control}$ for all *IL-15Rako* groups (BM MFI 723 vs 510, spleen 638 vs 730, LN 1115 vs 924, p=ns for all). CD25 remained slightly lower for T^{CXCR4} vs $T^{Control}$ (BM MFI 0 vs 50, spleen 0 vs 100, LN 0 vs 131, p=0.031, ns and 0.031 respectively).

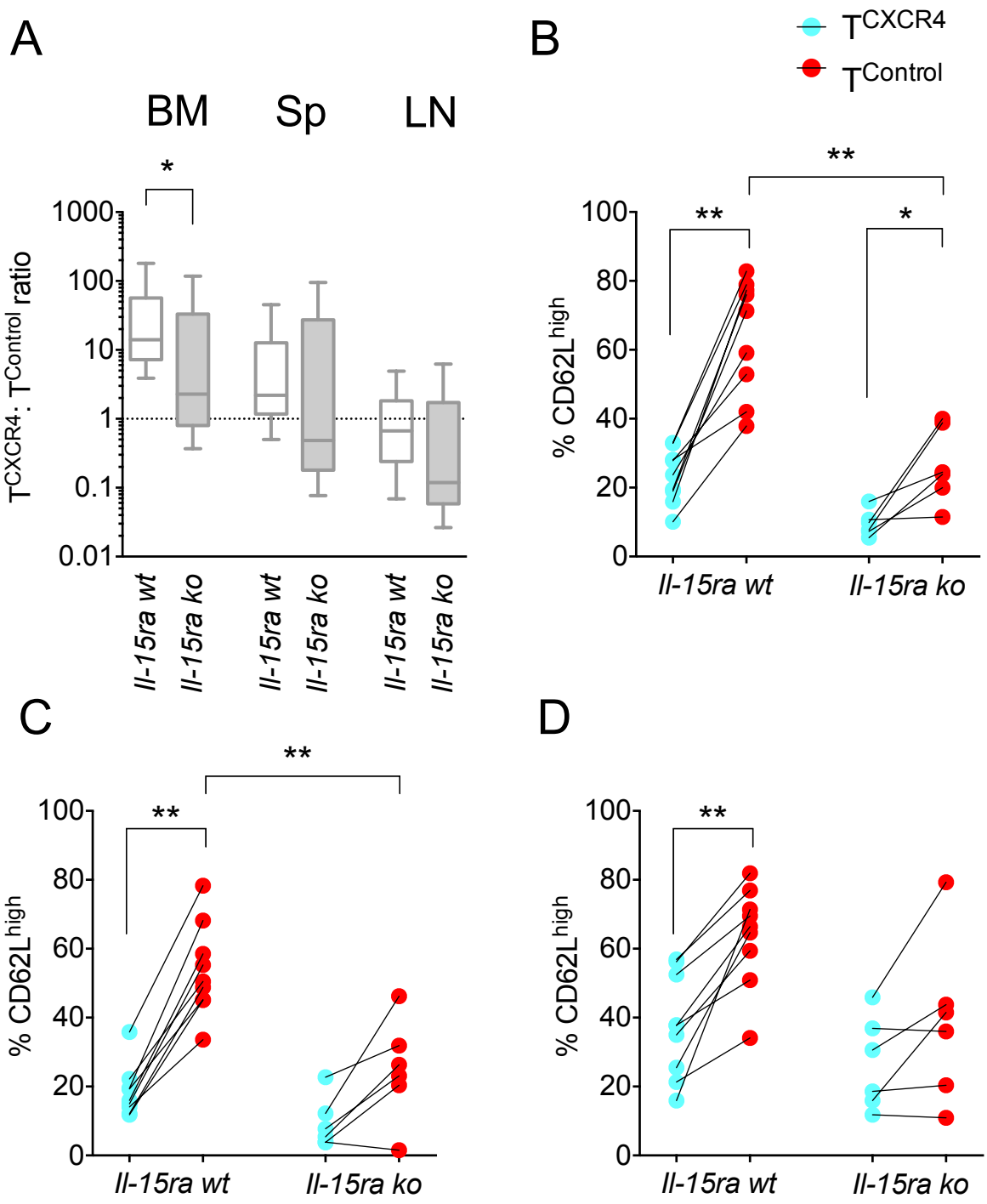

Figure 4-11 T^{CXCR4} BM homing and $CD62L^{high}$ phenotype are dependent upon IL15Ra in a vaccination model.

(A) Box and whisker plots denoting T^{CXCR4}: $T^{Control}$ ratio at day 36 in vaccinated *IL-15Ra wt* and *IL-15Rako* mice in BM, spleen and LN. Summary data showing the proportion of $CD62L^{high}$ T^{CXCR4} (red) and $T^{Control}$ (cyan) at day 36 in vaccinated *IL-15Ra wt* and *IL-15Rako* mice in (B) BM, (C) spleen and (D) LN. Mice were vaccinated as above with SIINFEKL-IFA at days 1 and 29 and organs harvested on days 8, 29 and 36. *P<0.05, **P<0.01, n = 15, 3 independent experiments.

Survival and Proliferation

Expression of the pro-survival protein BCL2 also became equivalent between T^{CXCR4} and $T^{Control}$ in vaccinated *IL-15Rako* mice. BM BCL2 positivity was markedly reduced at 8.1 for T^{CXCR4} vs 5.0% for $T^{Control}$ (Wilcoxon's signed rank; p=ns (Figure 4-12B) down from 52.4% in *Rag1ko* mice. Similarly, Spleen BCL2 positivity was 9.4% vs 6.3% (p=ns), down from 37.5% in *Rag1ko* mice. LN BCL2 %positive was 57.1% vs 54.5% (p=ns), down from 62.9%. BCL MFI showed a similar equivalence in *IL-15Rako* mice (BM MFI 396 vs 336, spleen 361 vs 342, LN 1297 vs 1324, p=ns for all). Reciprocally, expression of pro-apoptotic caspase 3 also became equivalent in all organs (BM caspase 3 MFI 504 vs 563, Figure 4-12C, spleen 642 vs 684, LN 836 vs 782, p=ns for all). T^{CXCR4} and $T^{Control}$ proliferated to a similar extent in vaccinated *IL-15Rako* mice (BM EdU MFI 3244 vs 4000, spleen 2333 vs 2754, LN 2025 vs 1848, Wilcoxon's signed rank; p=ns for all).

Senescence and exhaustion

The absence of IL-15Ra also rendered PD-1 and KLRG1 expression equivalent between splenic T^{CXCR4} and $T^{Control}$ (PD-1 %positive 29.8% vs 30.2%, PD-1 MFI 267 vs 242, KLRG1 MFI 45.4 vs 43.9, Wilcoxon's signed rank, p=ns for all) and there were no differences in the proportion of splenic MPECs and SLECs (MPECs 68.4% vs 32.9% and SLECs 2.6% vs 12.2%, p=ns (Figure 4-12D). The increased proportion of hyper-exhausted $T^{Control}$ cells (PD-1^{high}Eomes^{high}) seen in the *IL-15Ra wt* setting was also not seen in *IL-15Rako* mice (median 23.5% $T^{Control}$ vs 30.4% T^{CXCR4}, p=ns (Figure 4-12E).

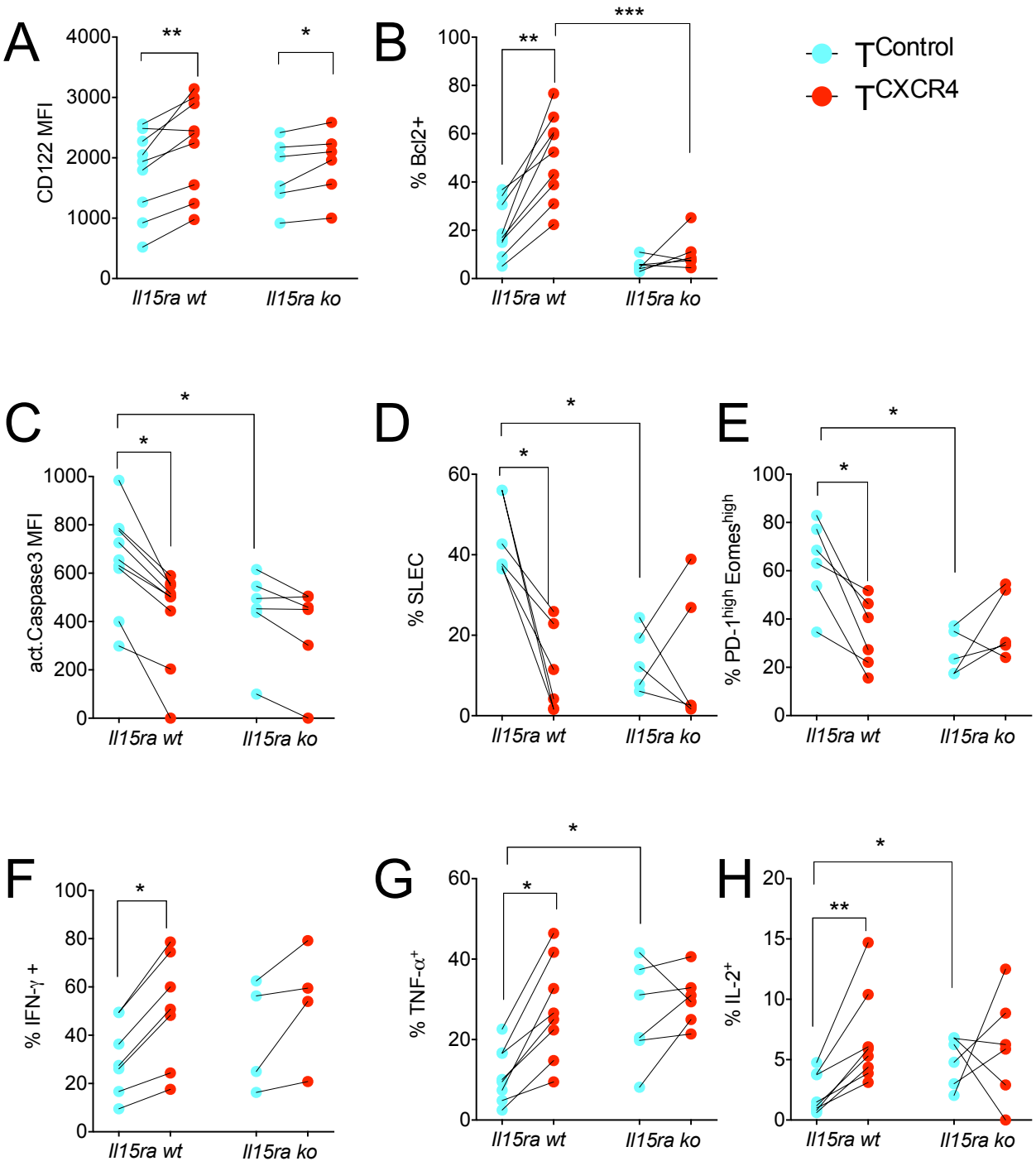

Figure 4-12 Absence of IL-15 signalling nullifies the T^{CXCR4} memory phenotype.

Summary T^{CXCR4} (red) and $T^{Control}$ data (cyan) at day 36 in vaccinated *IL-15Ra wt* and *IL-15Rako* mice for (A) BM CD122 MFI, (B) %Bcl2^{+}, (C) activated caspase 3 MFI, (D) splenic %SLEC and (E) %PD-1^{high}Eomes^{high} hyper-exhausted phenotype. (F-H) Summary data for cytokine production post ex vivo SIINFEKL stimulation of splenic T^{CXCR4} (red) and $T^{Control}$. *P<0.05, **P<0.01, ***P<0.001, n = 15, data pooled from 3 independent experiments.

Function

Post ex vivo splenocyte restimulation, there were no significant differences in the levels of cytokine expression between T^{CXCR4} and $T^{Control}$ in the absence of IL-15 signalling. IFN-γ% positive was 56.8% vs 40.7%, TNF-α 30.2% vs 25.8%, IL-2 6.1% vs 5.5%, (p=ns for all) (Figure 4-12F-H). The same was true for measurements by MFI (IFN-γ 692 vs 391, TNF-α 775 vs 673, IL-2 172 vs 147, p=ns for all).

IL-15Rako summary

In the absence of IL-15Ra, the defining T^{CXCR4} phenotypes of preferential bone marrow accumulation, T^{CM} formation, higher CD122 and BCL2 expression, and superior cytokine production are no longer seen. In addition, there are no longer any differences in markers of proliferation, senescence or exhaustion, suggesting that key differences in differentiation status between T^{CXCR4} and $T^{Control}$ are no longer present.

Two distinct phenotypic alterations were seen when comparing cells from *IL-15Ra wt* and *IL-15Rako* mice. For CD62L, BCL2 and CD122 expression, T^{CXCR4} lose their 'enhanced' phenotype to fall to similar levels as $T^{Control}$ (Figure 4-11B-D and Figure 4-12A-B). In contrast, for caspase 3 expression, formation of SLECs and hyper-exhausted phenotypes, the remaining $T^{Control}$ cells adopt the less terminally differentiated phenotype of T^{CXCR4} cells (Figure 4-12C-E). These conclusions are limited by numbers, but may be due to the fact that the tiny numbers of surviving $T^{Control}$ cells have managed to locate

alternative survival pathways, or represent a small proportion of memory cells with a long-lived phenotype.

4.6 In vitro T^{CXCR4} phenotype

An important question not yet addressed is the contribution of intrinsic CXCR4-CXCL12 signalling to the T^{CXCR4} phenotype. I investigated the in vitro T^{CXCR4} phenotype comparison to the $T^{Control}$ phenotype by culturing cells with recombinant CXCL12 under a number of different cytokine conditions. Because CXCL12 is present in serum at low concentrations, I also examined the effect of CXCR4 blockade with Plerixafor in vitro. The aim was to compare the in vitro phenotype with in vivo findings to determine to what degree the T^{CXCR4} phenotype resulted from intrinsic CXCR4-CXCL12 signalling, as opposed to extrinsic regulation by other factors. Thus, if a particular phenotype was seen to the same extent both in vitro and in vivo, and reversed by Plerixafor in vitro, this would suggest a dependence upon cell intrinsic signalling, as cell-extrinsic factors were lacking in my vitro model. In contrast, a phenotype seen solely in vivo would suggest a requirement for cell extrinsic signalling, possibly relating to the bone marrow microenvironment.

Previous experiments conducted by Dr. Ben Carpenter using transduced OT-I T cells showed that expression of CD44 and CD6L was equivalent between T^{CXCR4} and $T^{Control}$ after stimulation with relevant or irrelevant peptide, and the

presence or absence of 500 ng/ml murine CXCL12 did not alter phenotype, or IFN-γ generation following in vitro TCR activation. I therefore conducted a series of competition experiments exposing transduced polyclonal $T^{Control}$ and T^{CXCR4} to homeostatic cytokines during in vitro culture in the presence of varying levels of CXCL12. I examined the effect of prolonged culture over periods of 3-5 days post transduction upon memory phenotype, cytokine receptor expression and markers of survival and proliferation. Briefly, $CD8^+$ T cells were removed from transduction medium at day +3 post transduction, as per standard protocol, mixed in a 1:1 ratio, washed, and incubated with either IL-2 or a combination of IL-7 and IL-15 in T cell culture medium, in the presence or absence of CXCL12 or the CXCR4 antagonist Plerixafor.

Number, ratio, proliferation and survival

No differences were noted in phenotype at day 3 prior to 1:1 mixing. At day 5 post-transduction, numbers of T^{CXCR4} and $T^{Control}$ were comparable in wells where no additional CXCL12 was added (Baseline), regardless of the presence of IL-2 or IL-7 and IL-15 (median T^{CXCR4} number 38,877 vs $T^{Control}$ 51,752 in IL-2, 57,791 vs 51,699 in IL-7 and 15). Plerixafor did not alter absolute number or T^{CXCR4}: $T^{Control}$ ratio significantly (median T^{CXCR4} number 36,416 and $T^{Control}$ 45,414 in IL-2, 34,414 and 66,840 in IL-7 and IL-15). At low levels of CXCL12 (0.1 to 5 ng/ml), T^{CXCR4} and $T^{Control}$ numbers were similar to baseline. At higher concentrations of CXCL12 (100-1000 ng/ml) there was a progressive increase in absolute T^{CXCR4} number, whilst $T^{Control}$ number remained similar, resulting in an increased T^{CXCR4}: $T^{Control}$ ratio at high

CXCL12 levels (ratio 0.8 at baseline vs 2.2 at 1000 ng/ml CXCL12 with IL-2 and 0.9 at baseline vs 2.8 at 1000 ng/ml CXCL12 with IL-7 and IL-15 (Figure 4-13 and Table 3). Absolute T^{CXCR4} number trended towards correlation with increasing CXCL12 levels in the presence of homeostatic cytokines (Spearman's *rho* 0.59 for IL-2 and 0.80 for IL-7 and IL-15, 2 tailed t-test; p=0.09 and 0.014 respectively).

At higher CXCL12 levels there was a small increase in cellular proliferation for T^{CXCR4} in conjunction with IL-7 and 15, as measured by Ki67 staining (median Ki67 58.8% positive T^{CXCR4} at baseline vs 65.1% at 1000 ng/ml CXCL12 and $T^{Control}$ 47.2% positive at baseline vs 41.2% at 1000 ng/ml in IL-7 and IL-15, Figure 4-13). Ki67 proliferation showed a strong trend towards significant correlation with increasing CXCL12 levels for T^{CXCR4} regardless of cytokine conditions, but not $T^{Control}$ (Spearman's *rho* =0.66 and 0.65, p=0.05 and 0.06 for IL-2 and IL-7 and IL-15 respectively). No effect was seen for Plerixafor.

BCL2 expression was similar between T^{CXCR4} and $T^{Control}$ and did not change significantly upon exposure to CXCL12 in either cytokine condition (median MFI 1774 for T^{CXCR4} vs 2037 $T^{Control}$ at baseline and 2076 vs 1440 at 1000 ng/ml CXCL12 in IL-7 and 15).

Memory phenotype, CXCR4 and cytokine receptor expression

The majority of transduced cells maintained a $CD62L^{+}CD44^{+}$ central memory phenotype (T^{CM}) in culture and no differences were noted between T^{CXCR4} and $T^{Control}$ at baseline (median $\%T^{CM}$ 84.5 vs 84.7 respectively in IL-7 and IL-5

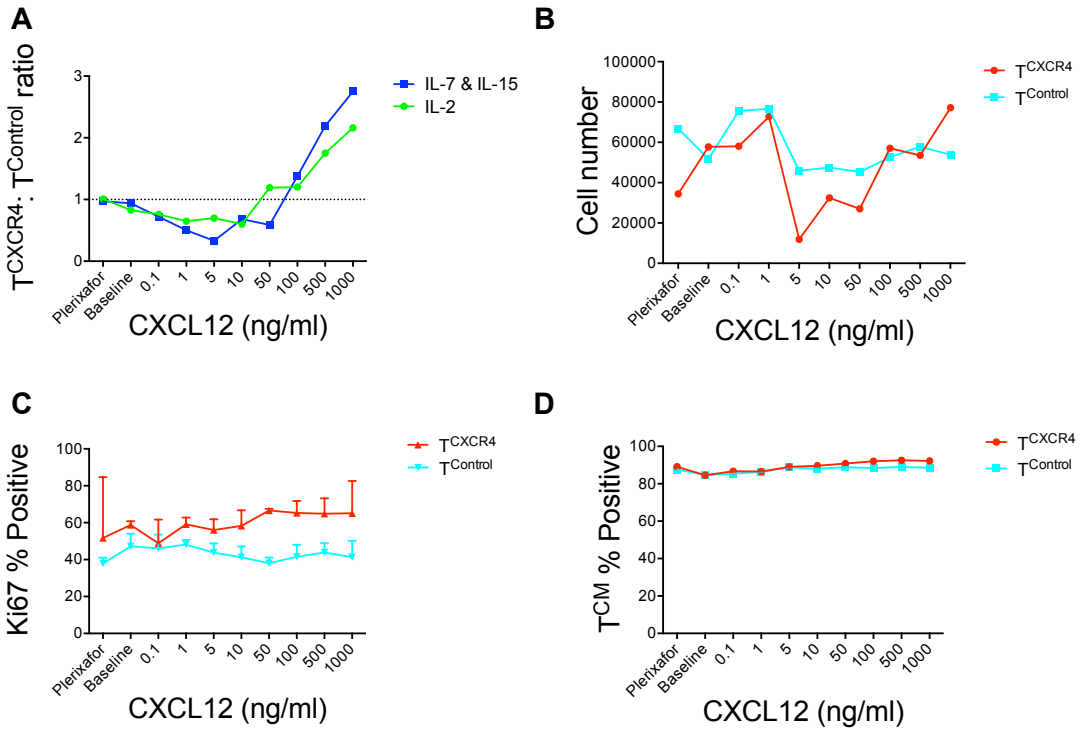

Figure 4-13 In vitro effects of CXCL12 upon cell number, proliferation and memory phenotype.
(A) Summary data for $T^{CXCR4:}$ $T^{Control}$ ratio at day 5 post transduction. Summary (B) absolute cell number (C) Ki67% positivity and (D) T^{CM}% for T^{CXCR4} and $T^{Control}$ at day 5 in wells containing IL-7 and IL-15 with Plerixafor or varying levels of CXCL12. Points and bars indicate median and inter-quartile range, data pooled from 4 independent experiments.

and 86.2 vs 81.9 in IL-2, p=ns for both) or at other CXCL12 concentrations (median $\%T^{CM}$ 92.2 vs 88.5 respectively in IL-7 and IL-5 and 91.4 vs 84.1 in IL-2 with 1000ng/ml CXCL12, p=ns for both) (Figure 4-13). CXCL12 concentration correlated with T^{CXCR4} $\%T^{CM}$ in IL-2, with a similar trend for IL-7 and IL-15 (Spearman's *rho* = 0.97 and 0.6, p<0.0001 and 0.08 respectively); however, the numerical difference was minimal. This effect was not seen in $T^{Control}$ cells and Plerixafor did not alter this phenotype.

CXCR4 expression on T^{CXCR4} correlated strongly with CXCL12 level in both IL-2 and IL-7 and IL-15 (Spearman's *rho* = -0.74 and -0.71, p=0.022 and

0.033 respectively (Figure 4-14) but not for $T^{Control}$ cells. Although median CXCR4 MFI on T^{CXCR4} reduced as CXCL12 level increased (2766 at baseline to 583 at 1000 ng/ml CXCL12 in IL-2), levels remained above those of $T^{Control}$ (baseline MFI 155 to 171 at 1000 ng/ml CXCL12 in IL-2). Plerixafor did not alter levels of CXCR4 expression under any conditions. No significant differences in cytokine receptor expression were seen between T^{CXCR4} and $T^{Control}$ at baseline (Figure 4-14 and Table 3) or at differing levels of CXCL12 and no correlations were seen between CXCL12 concentration and cytokine receptor expression.

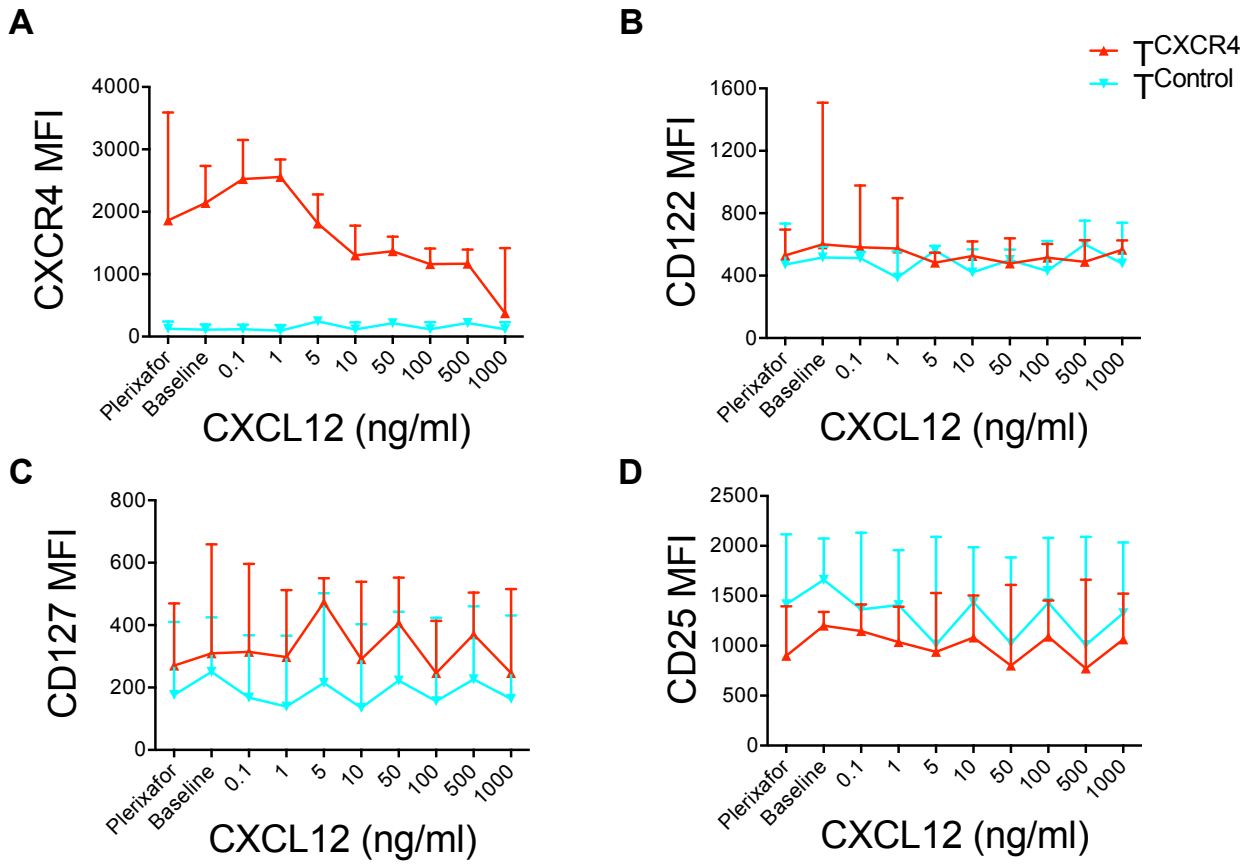

Figure 4-14 In vitro effects of CXCL12 upon CXCR4 and cytokine receptor expression.
Summary data at day 5 for (A) CXCR4 expression, (B) CD122, (C) CD127 and (D) CD25 expression for T^{CXCR4} and $T^{Control}$ in wells containing IL-7 and IL-15 with Plerixafor or varying levels of CXCL12. Points and bars indicate median and inter-quartile range, data pooled from 4 independent experiments.

	IL-2		IL-7 and IL-15		Correlation with CXCL12 concentration
	T^{CXCR4}	$T^{Control}$	T^{CXCR4}	$T^{Control}$	
Number	38877 (19366-195000)	51752 (35310-1060000)	57,791 (9484-672000)	51,699 (29600-114052)	T^{CXCR4} alone *IL-2: rho= 0.59, p=0.09* *IL-7 and IL-15: rho= 0.80, p=0.014*
% $Ki67^{+}$	61.1 (40.0-72.1)	58.4 (30.6-67.1)	58.8 (36.8-60.8)	47.2 (42.2-54.0)	T^{CXCR4} alone *IL-2: rho= 0.66, p=0.05* *IL-7 and IL-15: rho= 0.65, p=0.06*
% T^{CM}	86.2 (71.6-90.2)	81.9 (79.8-85.0)	84.5 (80.0-87.6)	84.7 (77.3-85.3)	T^{CXCR4} alone *IL-2: rho= 0.97, p<0.0001* *IL-7 & IL-15: rho= 0.60, p=0.08*
CXCR4 MFI	2766 (515-3575), p<0.001	155 (35-161)	2140 (567-2734), p=0.045	113 (31-194)	T^{CXCR4} alone *IL-2: rho= -0.74, p=0.022* *IL-7 & IL-15: rho= -0.71, p=0.033*
BCL2 MFI	1983 (1405-2387)	1842 (1532-2992)	1774 (1421-1777)	2037 (2025-2871)	No
IL-2/15Rβ MFI (CD122)	570 (370-728)	423 343-576)	601 (461-1784)	517 (317-596)	No
IL-7Rα MFI (CD127)	486 (149-712)	765 (153-1130)	310 (113-712)	250 (108-436)	No
IL-2Rα MFI (CD25)	1380 (828-1702)	1295 433-2144)	1202 (923-1340)	1660 (541-2123)	No

Table 3 Summary of in vitro data for T^{CXCR4} and $T^{Control}$ at baseline. Median (ranges) values shown for all, data pooled from 4 independent experiments, p=ns except when shown.

In vitro summary

No difference was seen between T^{CXCR4} and $T^{Control}$ in wells where no exogenous CXCL12 was added, or Plerixafor was used, suggesting that the effect of CXCL12 signalling at baseline is minimal due to insufficient quantities of CXCL12 in T cell culture medium. Low exogenous CXCL12 levels also had minimal effects upon cellular proliferation or cell number. At high CXCL12 concentrations (>100 ng/ml), cell number and proliferation were specifically enhanced in T^{CXCR4}, enabling a tiny advantage over $T^{Control}$ cells at the highest level. This difference did not relate to cell survival, as shown by unaltered BCL2 expression, and was unaffected by the choice of cytokine.

Increasing CXCL12 had a similarly tiny effect in increasing the proportion of T^{CXCR4} with a central memory phenotype. As expected, high CXCL12 levels had the effect of reducing CXCR4 expression in T^{CXCR4} 4-fold from baseline due to receptor internalisation, but T^{CXCR4} at 1000 ng/ml retained 5-fold higher expression than $T^{Control}$, suggesting that prolonged high level CXCR4 signalling can persist despite receptor downregulation. None of these findings were affected by homeostatic cytokine addition, which may relate to localised concentration gradients and IL-15 trans-presentation causing differing effects within the complex in vivo BM microenvironment.

4.7 Discussion

The data presented above demonstrate that T^{CXCR4} maintain a dramatically increased numerical advantage compared to $T^{Control}$ in bone marrow and spleen after boost vaccination, with greater proportions of T^{CM}, IL-15Rβ and BCL2 expression in all organs, enhanced levels of effector cytokine expression upon restimulation, and higher frequencies of polyfunctionality. One of the interesting differences between T^{CXCR4} and $T^{Control}$ was the ability of a proportion of T^{CXCR4} to produce IL-2. This feature has an autocrine role in enhancing IFN-γ production independent of proliferation in protective immune responses (489) and T cells with high IL-2: IFN-γ ratios demonstrate superior long-term persistence and self-renewal in patients with metastatic melanoma (490).

One potential concern with efforts to alter the phenotypic balance to generate memory T cells, for example by inhibition of the mTOR signalling pathway, is the production of cells with limited expansion and effector capability (491). In our model CXCR4 over-expression led to enhanced bone marrow and splenic accumulation at early time points post vaccination and subsequently superior numbers of T^{CXCR4} in all organs at later time points, suggesting that substantial trafficking of T cells from the bone marrow niche and enhanced bone marrow persistence allows seeding of other tissues. Consistent with the multipotent profile of T^{CM}, T^{CXCR4} also produced higher number of $T^{EM/EFF}$ cells at day 36, indicating proficiency in recapitulating the entire immune repertoire.

Consistent with an early memory phenotype, T^{CXCR4} are characterised by higher frequencies of a memory-associated $Eomes^{High}$ $T\text{-}bet^{Low}$ transcription

factor profile and express very low levels of the inhibitory exhaustion marker PD-1 and terminal senescence marker KLRG1 despite repeated antigenic stimulation. In contrast, the in vitro model failed to demonstrate major differences between T^{CXCR4} and $T^{Control}$ in terms of memory phenotype, expression of cytokine receptor expression, apoptosis-associated proteins, or production of effector cytokines. Any differences seen in number seemed to relate to higher levels of proliferation seen at high CXCL12 concentrations, conflicting with the in vivo phenotype.

Coupled with the findings of the *IL-15Rako* experiments, these differences suggest that the T^{CXCR4} phenotype is predominantly maintained by extended in vivo exposure to extrinsic factors, in particular IL-15 signalling. As the BM is a site of superior IL-15 bioavailability, this would explain the greater tendency towards this phenotype seen in the *IL-15Ra.wt* BM niche compared to other organs. The incomplete abolition of this phenotype in the BM of *IL-15Rako* mice suggests that other factors such as IL-7 may play a minor role, or the persistence of residual IL-15 signalling.

The polyfunctional T^{CM} profile suggests that T^{CXCR4} possess superior tumour control abilities on a per-cell basis, as implied by data from the tumour model. We did not see evidence of the rare T^{SCM} subset in our experimental model after antigen-exposure, highlighting experimental difficulties in isolating this subset without pharmacological manipulation. Although T^{CXCR4} were $CD122^{high}$ and $BCL\text{-}2^{high}$, they were also uniformly $CD44^{high}$ and Sca-1 expression levels were no greater than $T^{Control}$. This finding may relate to the extensive antigen exposure in our OT-I model, TCR avidity or the degree of activation and exposure to IL-2 involved in the retroviral transduction protocol.

5 The effect of transient CXCR4 overexpression upon T cell phenotype and function

5.1 Introduction

I have shown that constitutive high level CXCR4 expression produces a pronounced T^{CM} phenotype in vivo. Continuous expression may have undesirable effects such as excessive retention in particular niches and is less tractable to the demands of clinical translation and the requirements of different immunotherapeutic strategies. An approach employing transient overexpression might achieve similar phenotype imprinting without potential negative effects. This is based upon the concept that transient exposure to factors such as IL-15 responsible for the early memory phenotype would be sufficient to determine long-term cell fate and implies that long-term exposure is not required.

This idea is in keeping with models suggesting that conditions during initial antigen priming and memory generation are more important in determining cell fate than the later stages of memory homeostasis (492). This may also be complementary with the concept that continuous intrinsic CXCL12 signalling is not required for the maintenance of this phenotype. This approach is potentially more easily employable as concerns over continuous CXCL12 signalling in a long-lived stem-like cell might hinder clinical translation. In the next set of experiments, I therefore tested the hypothesis that transient overexpression of CXCR4 would be sufficient to convey an early memory

phenotype upon transduced T cells. I used a model where CXCR4 expression can be switched on and off through exposure to the commonly available antibiotic doxycycline. In the 'Tet-ON' retroviral system the gene of interest (CXCR4 marked with GFP or GFP alone for control cells) is under control of a Tet-Operon rendering the promoter transcriptionally inactive.

The vector, which was created and validated by Dr Pedro Velica in our lab, also encodes the reverse-tetracycline-controlled transactivator (rtTA2-M2), marked by the Q8 surface tag (Figure 5-1A). In the absence of doxycycline, the promoter is inactive and the transduced cells express Q8 alone. In the presence of doxcycline, rtTA2-M2 binds to the Tet Operon and promotes CXCR4 and GFP transcription (493). Removal of doxycycline from the system reinstates repression, switching off CXCR4 and GFP expression. The vector was validated in vitro. 24 hours post transduction, $CD8^+$ T cells were divided into 2 flasks and doxycycline was added to 1 flask at 0.5 µg/ml. Cells were analysed 72 hours post transduction, and CXCR4 expression for iCXCR4 No Doxycycline and iCXCR4 Doxycycline were compared to mock transduced and constitutive CXCR4-transduced cells. As shown in Figure 5-1B below, iCXCR4 No Doxycyline express similar low levels of CXCR4 to mock transduced cells. Induced CXCR4 expression as measured by flow cytometry increased MFI level to approximately 40% of the constitutive vector.

My hypothesis consisted of two parts. Firstly, transient CXCR4 expression in a polyclonal setting would recapitulate the constitutive polyclonal phenotype in the short term in the absence of antigen priming. Secondly, that this phenotypic advantage over iGFP could also be established and maintained in the primary-boost model after CXCR4 expression had been switched off.

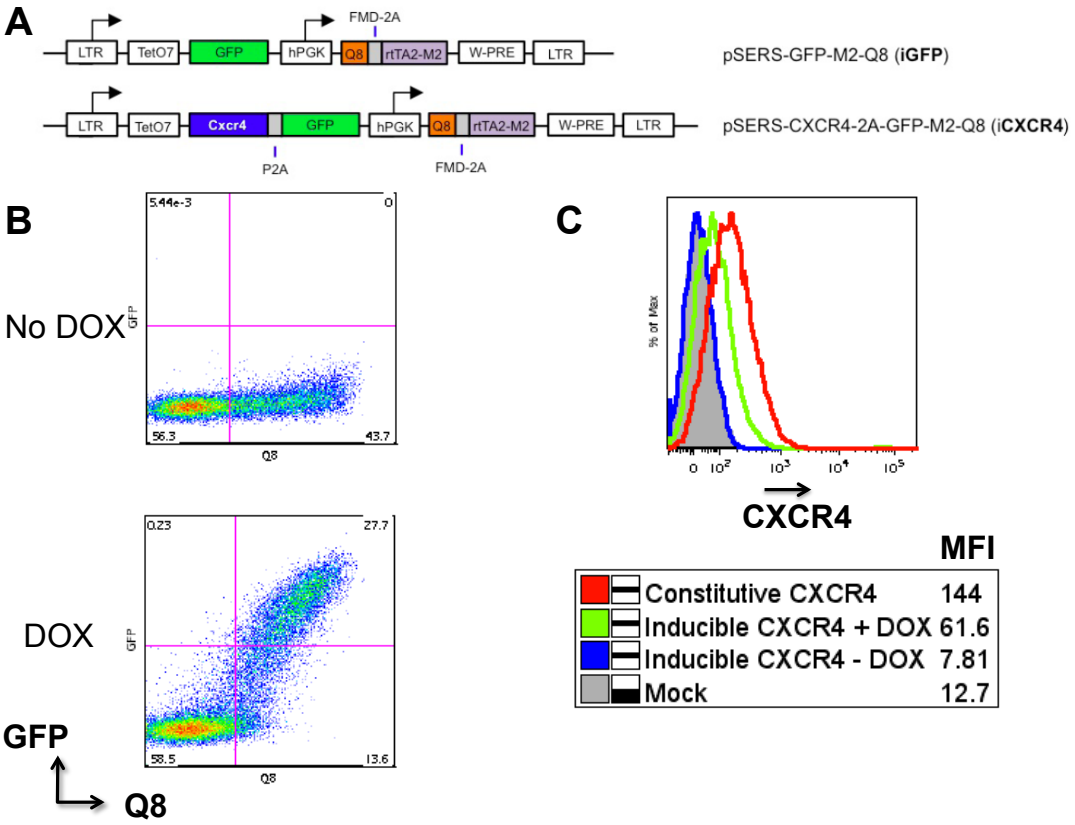

Figure 5-1 iCXCR4 vector map and flow cytometry validation.
(A) iGFP and iCXCR4 doxycycline-inducible vector maps: Long Terminal Repeat (LTR); Tetracycline operator (TetO7); Foot-and-mouth disease 2A sequence (FMD-2A); Human phosphoglycerate kinase (hPGK); optimized reverse tetracycline-controlled transactivator (rtTA2-M2); Woodchuck hepatitis Post-transcriptional Regulatory Element (W-PRE); Picornavirus 2A sequence (P2A). (B) At 24 hours post iCXCR4-transduction $CD8^+$ T cells were cultured in the absence (No DOX) or presence of doxycycline (DOX). Cells were analysed by flow cytometry 48 hours later for expression of Q8 surface marker of transduction and GFP. (C) CXCR4 expression 72 hours post-transduction for constitutive CXCR4-transduced cells, Q8-positive doxycycline and no doxycycline iCXCR4 and mock-transduced cells. Figures indicate MFI.

5.2 Testing the effect of transient CXCR4 over-expression upon memory generation in a polyclonal model

In the first set of experiments I used a polyclonal model for comparison with the polyclonal constitutive CXCR4 phenotype, as this setting is more analogous to the clinical setting, and reflects the polyclonal repertoire of gene-engineered cells used in most clinical trials. Bead-sorted activated polyclonal $CD8^+$ T cells from B6 splenocytes were transduced with the inducible CXCR4 construct (iCXCR4) or the inducible GFP construct (iGFP). iCXCR4 and iGFP cells were mixed in a 1:1 ratio of Q8-positive cells and injected into irradiated B6 recipients. Mice were divided into 3 groups; those exposed to doxycycline continuously for the length of the experiment (Continuous), those exposed transiently from Day -1 to Day 8 (Transient), and those never exposed (Non-exposed) (Figure 5-2). I used a Continuous group to establish if the lower level of CXCR4 expression seen with the inducible vector resulted in differences in phenotype compared to the constitutive vector. As before I investigated accumulation and memory phenotypes in lymphoid organs. On the day of injection (day 0) iCXCR4 and iGFP were no different in terms of T^{CM} frequency (median 75.9 vs 80.6%) or cytokine receptor expression.

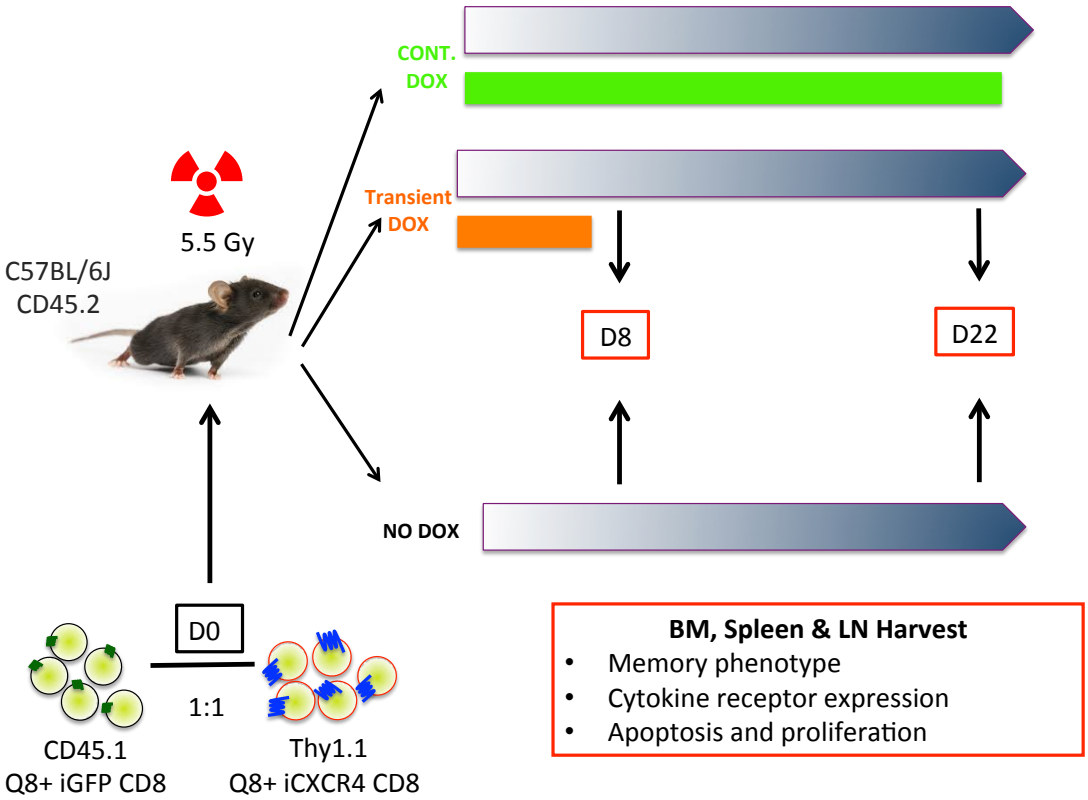

Figure 5-2 Transient CXCR4 polyclonal model.
Congenically distinct transduced iGFP and iCXCR4 polyclonal B6 $CD8^+$ T cells were mixed in a 1:1 ratio and transferred into irradiated recipients. Mice were exposed to doxycycline continuously, transiently or not at all and organs were harvested for assessment at days 8 and 22 post injection. Data representative of 2 independent experiments, n=10.

Number and ratio

On day 8 post injection, in the Continuous group, there were increased numbers of iCXCR4 compared to iGFP in the bone marrow (median ratio 2.4:1 and reduced levels in spleen and LN (median ratios 0.8:1 and 0.3:1 respectively). On day 22 post injection in the Continuous group, there was again slightly increased BM accumulation for iCXCR4: iGFP (median ratio 1.4:1) and reduced accumulation in spleen and LN (ratio 0.65:1 for both). A similar pattern was seen in the Transient group, with a tendency to increased BM accumulation (median ratio 3.4:1) and reduced ratios in the spleen and LN (0.3:1 and 0.4:1 respectively).

Memory and survival phenotype

On day 8, there were no consistent differences in central memory phenotype between iCXCR4 and iGFP (median T^{CM}% 44.0 vs 45.1, 41.4 vs 33.9 and 42.3 vs 37.9 in BM, spleen and LN respectively).

However, by day 22, iCXCR4 in the Continuous group showed a similar central memory skewing to the constitutive vector phenotype in all organs (BM iCXCR4 vs iGFP T^{CM} 66.4% vs 19.0%, spleen 74.8% vs 29.3%, LN 78.5% vs 68.0%). In contrast, iCXCR4 in the Transient group showed no differences from iGFP controls (BM T^{CM} 9.3% vs 7.3%, spleen 36.0% vs 26.0%, LN 54.9% vs 57.2%).

IL-15Rβ expression remained similar at day 8 and 22 in all groups (day 8 BM MFI 395 vs 379, day 22 Continuous BM 421 vs 295, day 22 Transient BM 356

vs 354). Finally, there was a tendency towards enhanced expression of BCL2 for iCXCR4 vs iGFP in the Continuous group at day 22 only (BM MFI 380 vs 340, spleen 801 vs 571).

Summary

Continuous CXCR4 expression using the inducible vector did not initially enable a memory phenotypic advantage in iCXCR4 compared to iGFP, but did enable a later advantage at day 22, and as expected showed a BM accumulation advantage at both time points. All the features of the constitutive phenotype were not recapitulated in terms of IL-15Rβ expression and BCL2 expression however the limited number of replicates did not allow for statistical comparison. Similarly, although there was enhanced BM accumulation for the Transient group, this did not confer any enhancement of memory phenotype. In response to the first part of my hypothesis, transient CXCR4 expression in a polyclonal setting did not recapitulate in full the constitutive polyclonal phenotype in the short term in the absence of antigen priming. I therefore sought to shed light upon these inconclusive preliminary findings by evaluating the inducible vector further in the vaccination setting.

5.3 Testing the effect of transient CXCR4 over-expression upon memory generation in the vaccination model

Having established some similarities between the phenotype of polyclonal T cells transduced with inducible and constitutive vectors, I then extended investigation of the inducible phenotype into the primary-boost vaccination model. For comparison purposes with the constitutive vaccination experiments I used *Rag1ko* mice. Owing to difficulty identifying the Q8 surface tag by flow cytometry in some organs, I labeled and flow-sorted Q8 positive-transduced OT-I $CD8^+$ T cells on the day of injection to aid identification of iCXCR4 and iGFP. iCXCR4 and iGFP Q8-positive cells were mixed in a 1:1 ratio before injection (see Figure 5-3). T^{CM} frequency of sorted iCXCR4 and iGFP cells were similar on day 0 (median 53.1% vs 58.3% respectively).

Mice were vaccinated with SIINFEKL-IFA on days 1 and 29 and divided into three groups (Continuous, Transient and Non-exposed). As before, CXCR4 and GFP expression were induced in vivo by oral administration of doxycycline to mice for day -1 to day 8 (Transient) or day -1 to day 36 (Continuous).

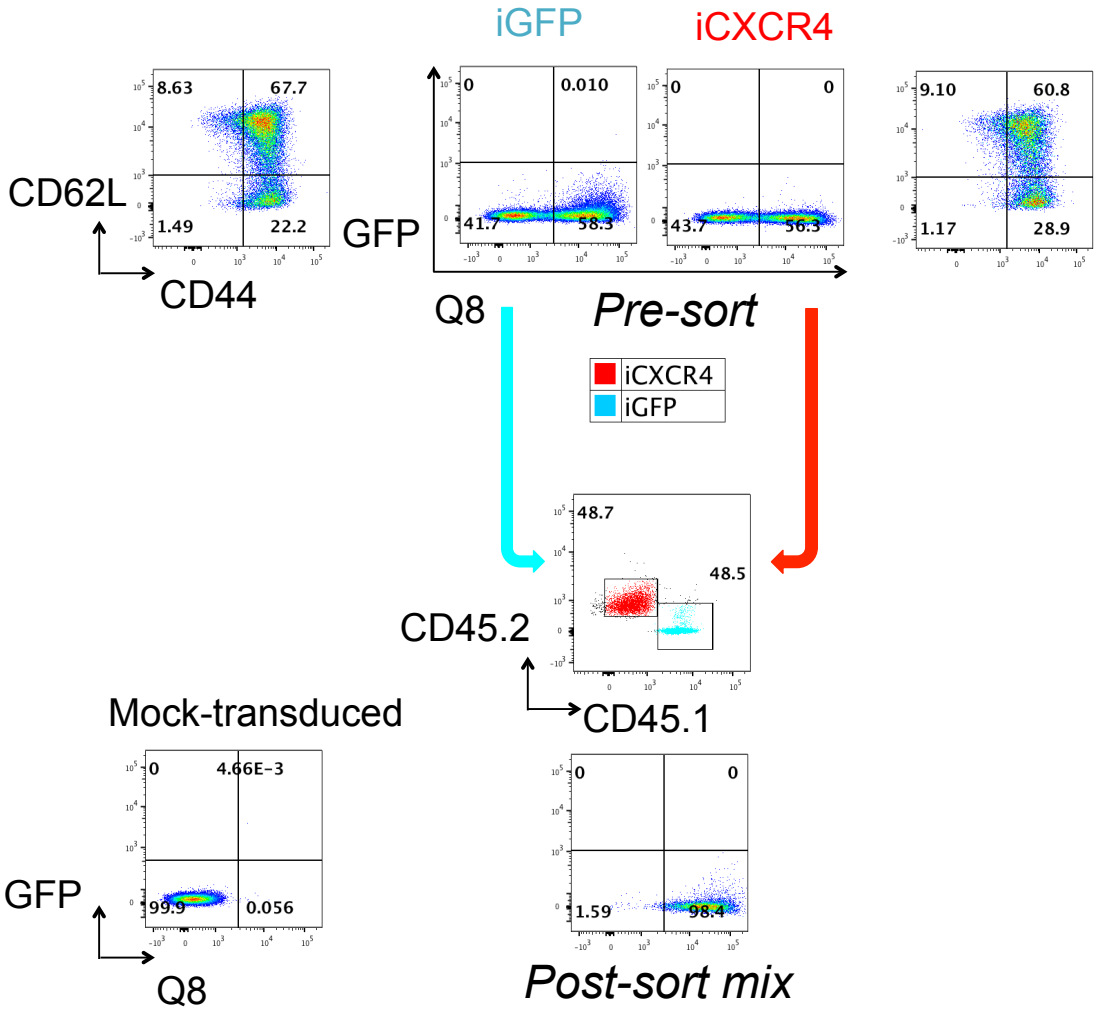

Figure 5-3 Q8 Sort strategy.
iGFP (cyan) and iCXCR4 (red)-transduced CD8$^+$ T cells were stained and flow-sorted for Q8 positivity at 72 hours post-transduction, mixed in 1:1 ratio and injected into recipients. Flow plots gated on live CD8$^+$ T cells show pre and post-sort Q8-GFP plots, central memory frequencies for iCXCR4 and iGFP, 1:1 mix by congenic marker and mock-transduced cells.

Number and ratio

At day 8, the median ratio of Q8+GFP+ iCXCR4: iGFP was increased in BM at 2.1:1 and reduced in spleen and LN (ratios 0.6:1 and 0.4:1, p=0.074, 0.002 and 0.049 respectively). Median numbers were 6027:2752 in BM, 6810:12678 in spleen and 1066 vs 2581 in LN, p=ns for all (Figure 5-4A). At day 29,

absolute numbers were markedly increased in BM in the Continuous group (286,356: 2464, ratio 116:1), and also in spleen (170556:11051, ratio 15.4:1)

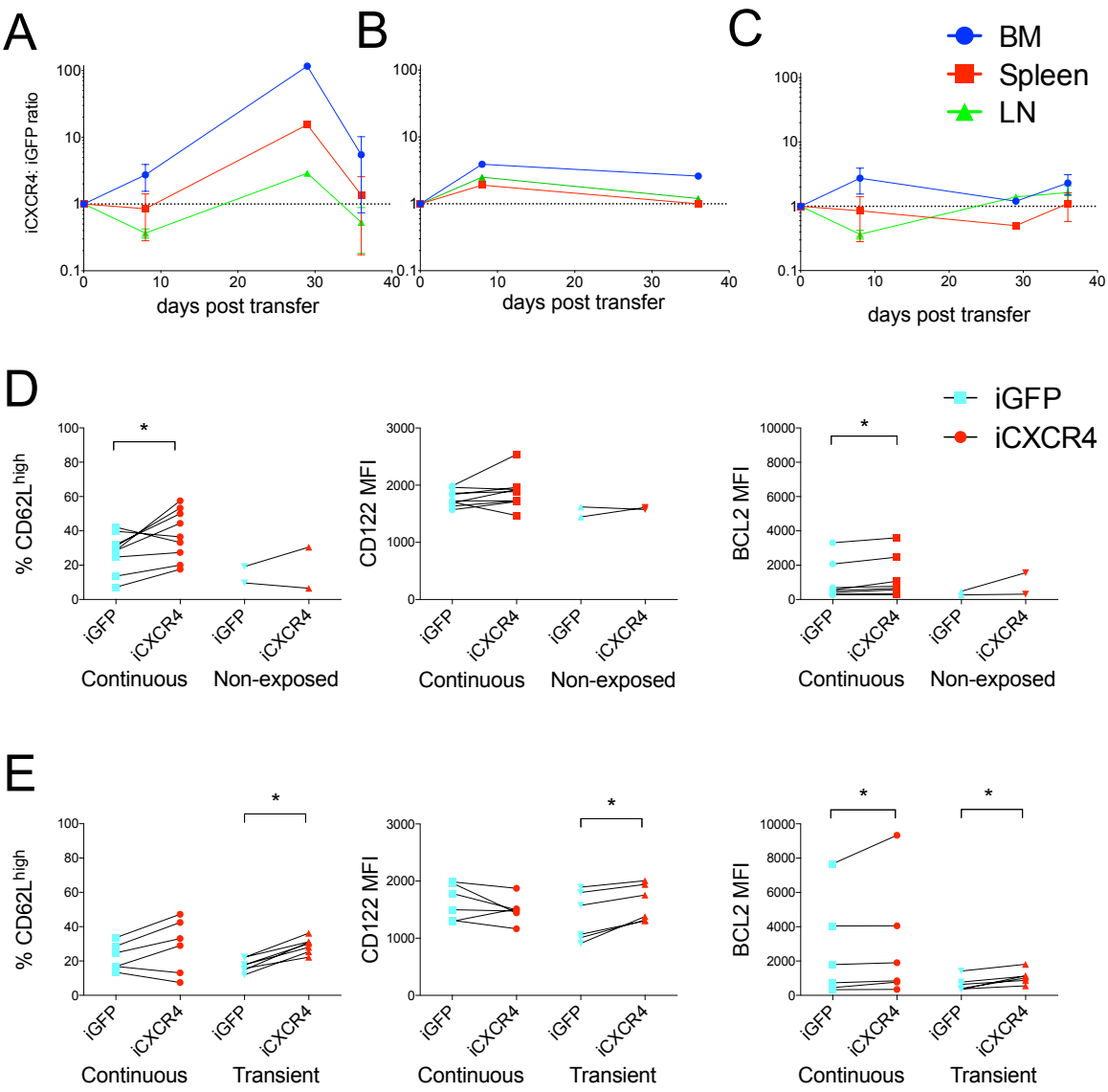

Figure 5-4 iCXCR4 kinetics and phenotype post vaccination.
Summary data for iCXCR4: iGFP ratio in (A) the continuous doxycycline group, (B) the transient and (C) non-exposed doxycycline groups. Data representative of one experiment, n=14. (D) Day 8 central memory frequency, CD122 and BCL2 MFI for iCXCR4 and iGFP exposed or not to continuous doxycycline. Data representative of two independent experiments, n=11, statistical significance tested using the 2-tailed paired t-test. (E) Day 36 central memory frequency, CD122 and BCL2 MFI for iCXCR4 and iGFP exposed to continuous or transient doxycycline. Data representative of two independent experiments, n=14, statistical significance tested using the Wilcoxon ranked sum test, *P<0.05.

and LN (6460:2213, ratio 2.9:1). In contrast numbers were similar in the Transient group at day 29 in BM (8323:6958, ratio 1.2:1) and LN (2528:1771, ratio 1.4:1), and reduced in the spleen (28087:51242, ratio 0.5:1) (Figure 5-4A & B).

At day 36, following boost vaccination in the Continuous group, absolute numbers were increased in BM (7845:2613, ratio 4.2:1), similar in spleen (45785:46327, ratio 1.0:1.0) and reduced in LN (1344:2650, ratio 0.5:1) (Figure 5-4A). In the Transient group, numbers were slightly enhanced in BM (20757:6786, ratio 2.3:1) and similar in spleen and LN (108742:63414 and 14239:6925, ratios 0.8:1 and 1.6:1 respectively) (Figure 5-4B). Ratios in the Non-exposed group were similar to the Transient group at 2.6, 1.0 and 1.2 in BM, spleen and LN respectively, n=1 (Figure 5-4C).

Memory phenotype

At day 8, there were small increases in T^{CM} frequency for iCXCR4 over iGFP in all organs (BM median 36.5% vs 28.6%, spleen 37.8% vs 24.9%, LN 62.2% vs 51.2%; p=0.036, 0.006 and 0.016 respectively). T^{CM} frequencies in the Non-exposed group at day 8 were 18.5 vs 14.5, 16.6 vs 14.8 and 36.1 vs 36.7 for BM, spleen and LN respectively, n=2 (Figure 5-4D).

At day 36 in the Continuous group however, there were no significant differences in the frequencies of T^{CM} for iCXCR4 vs iGFP in any organs (BM median 31.1 vs 20.8, spleen 14.0 vs 10.1, LN 43.9 vs 36.5; p=ns for all). In contrast, there was a modest increase in the Transient group iCXCR4 T^{CM}

frequency over iGFP for all organs (BM% 29.1 vs 16.8, spleen 19.7 vs 11.3 and 44.5 vs 29.0; p=0.016, 0.031 and 0.016 respectively) (Figure 5-4E). In the same pattern as for cell number however, iCXCR4 T^{CM} frequencies were similarly increased in the Non-exposed group at 36.2 vs 22.1, 27.6 vs 11.6 and 33.0 vs 18.4 for BM, spleen and LN respectively.

Cytokine receptors

At day 8 there were no significant differences between IL-15Rβ expression for iCXCR4 vs iGFP in any organs (BM MFI 1888 vs 1725, spleen 1548 vs 1431, LN 1546 vs 1439) (Figure 5-4D). The same was true of CD127/132/25.

At day 36, IL-15Rβ expression was again similar in the Continuous group for iCXCR4 vs iGFP (BM MFI 1487 vs 1642, spleen 1190 vs 1332, p=ns for all) (Figure 5-4E). Transient group iCXCR4 IL-15Rβ expression was slightly higher in both BM and spleen (BM MFI 1567 vs 1322, spleen 1154 vs 1078, p=0.031 for both, insufficient cells for LN analysis) (Figure 5-4E). Expression in the Non-exposed group was 1503 vs 1271 for BM and 715 vs 982 for spleen.

Survival and proliferation

At day 8, there was a small increase in BCL2 expression for iCXCR4 over iGFP (BM, spleen and LN median BCL2 MFI 655 vs 518, 779 vs 550 and 768 vs 668; p=0.020, 0.009 and 0.028) (Figure 5-4D). Caspase 3 expression was

not different in any organ. A similar pattern was also noted in the Non-exposed group: BM BCL2 MFI was 936 vs 377, spleen 2422 vs 2040, LN 1053 vs 637 in LN.

At day 36 in the Continuous group, iCXCR4 expressed slightly increased BCL2 levels in BM and spleen (BM MFI 1379 vs 1265 and spleen 769 vs 603, p=0.031 for both) and similar in LN (MFI 1517 vs 1153, p=ns). In the Transient group, BCL2 expression was higher for iCXCR4 vs iGFP in all organs (BM MFI 1065 vs 518, spleen 1173 vs 1001, LN 1548 vs 990, p=0.031 for all) (Figure 5-4E). In the Non-exposed group, BCL2 MFI was 8936 vs 8512 in BM, 2789 vs 2875 in spleen and 7668 vs 7557 in LN.

Caspase 3 expression was similar between groups in all organs at day 36, other than a slight increase in spleen in the Transient group ((MFI 454 vs 260, p=0.031). Proliferation was similar for iCXCR4 vs iGFP in BM and spleen post primary and boost vaccinations (day 8 EdU MFI in BM 6933 vs 7215, spleen 5889 vs 6315, p=ns for both).

Function

At day 8, IFN-γ release from stimulated splenocytes was significantly lower in iCXCR4 vs iGFP (IFN-γ MFI 1674 vs 3369; p=0.008) and TNF-α release was equivalent (TNF-α MFI 613 vs 454, p=ns). At day 36, IFN-γ MFI again trended lower for iCXCR4 vs iGFP in the Continuous group and was equivalent for the Transient group (MFI 0 vs 40.4 and 0 vs 10, p=0.063 and ns respectively). No differences were seen for TNF-α production.

5.4 Discussion

In answer to the second part of my hypothesis, a relevant memory phenotypic advantage for iCXCR4 over iGFP was not seen in the primary-boost vaccination model after CXCR4 expression was switched off. Firstly, although small differences were seen in T^{CM} accumulation, CD122 and BCL2 expression at day 8 and day 36 in the Transient group, these differences were not of the same magnitude as seen in the constitutive vaccination setting. Indeed, the degree of T^{CM} frequency and increased BM accumulation was similar to that seen in the Non-exposed group. This suggests that the negative control in this experiment (the Non-exposed group) did not function as required and that absence of doxycycline incompletely repressed CXCR4 expression. The reverse tetracycline transactivator system (rtTA) is known to display problems with 'leaky' target gene transcription and expression in the absence of tetracycline, despite use of a vector designed to reduce this issue (494). The rtTA system undergoes a conformational change in the presence of tetracycline, increasing affinity of the activation domain for a DNA binding site and enabling dramatic enhancement of transcription. Leakiness is dictated by basal affinity retained by the rtTA for the DNA binding site even in the absence of doxycycline. It may have been that a small increase in the number of CXCR4 molecules expressed on the T cell surface was enough to offer a competitive BM accumulation advantage over iGFP cells. Recent studies have suggested techniques for mitigating against this unwanted transcription by use of single amino acid substitutions and sensitivity-enhancing mutations, reducing leakiness to undetectable levels (494).

Continuous CXCR4 expression induced by doxycycline was able to enhance BM accumulation of iCXCR4 over iGFP in competition assays using both polyclonal and antigen-specific T cells in a vaccination model. However iCXCR4 did not show the preferential splenic accumulation seen with the constitutive vector. Importantly, continuously induced CXCR4 expression over the duration of the experiment failed to recapitulate the constitutive vector early memory phenotype in terms of markedly increased T^{CM} frequency, IL-15 receptor and BCL2 expression or cytokine production. As shown in Section 4.4 and Figure 4-10, level of CXCR4 expression correlates with memory phenotype, hence these findings probably relate to the lower level of CXCR4 expression of CXCR4 produced by the inducible vector.

The Continuous group is the positive control in the experiment, but this finding renders interpretation difficult. The fact that enhanced BM accumulation is maintained suggests that sufficient CXCR4 is expressed to enhance homing in a similar way to the constitutive vector, but that lower level expression relative to the constitutive vector may prevent formation of advantageous memory features. The concept of this 'threshold' effect is supported by the findings of reduced BCL2 expression in T^{CXCR4} expressing lower levels of CXCR4 in the constitutive primary-boost model, and indirectly by imaging findings showing that higher CXCR4 expression alters dynamic T cell behaviour by reducing track straightness (Section 3.2).

To summarise, in their current state these retroviral tools do not allow us to answer the question of whether transient CXCR4 expression is sufficient to recapitulate the CXCR4 memory phenotype seen with the constitutive vector.

They also reiterate the requirement for high-level CXCR4 expression to produce marked changes in T cell memory phenotype.

Further experiments are required to answer the hypotheses using vectors that inducibly enable higher levels of CXCR4 expression, and it would be interesting to assess if we could ‘tune’ expression level to achieve the desired phenotype. We used a transient expression period of 8 days in these experiments, to assess whether expression at the time of priming could enhance memory formation. The second question therefore is whether this expression period is too short, and a period of 4-5 weeks is required, as seen in the constitutive primary-boost model. Continuous expression using the inducible vector for 5 weeks was able to enhance BM accumulation progressively over time in the vaccination setting, in a very similar pattern to the constitutive model, so further experiments could be employed to vary the period of transient expression. It is particularly important to examine if re-expression at time of boost vaccination is important. This question feeds into the issue of how much exposure to putative niche-mediated factors such as IL-15 is required to mediate memory phenotype. Continuous or repeated exposure may hinder the translational applicability of the CXCR4 concept.

6 Pharmacological blockade and induction of bone marrow homing

6.1 Introduction

Thus far, I have demonstrated that constitutive over-expression of CXCR4 in $CD8^+$ T cells enhances accumulation in the bone marrow and functional memory characteristics. In order to examine if this memory phenotype was purely dependent upon BM homing per se, I hypothesised that alternative methods of BM homing, namely (a) physical emplacement of T cells within the BM and (b) pharmacological enhancement, would replicate this phenotype.

If these hypotheses were proven correct, this would potentially offer a more straightforward and cost-effective means of producing a similar phenotype for quicker clinical translation, without the regulatory hurdles of genetically modified immune cells. If not, this would suggest that the requirement for high-level CXCR4 expression might enable preferential homing to a particular niche, which is not replicated by alternative methods. This finding would be potentially important for future exploration of the underlying mechanism and clinical development of this approach. I firstly investigated if the effect of CXCR4-mediated bone marrow homing could be replicated by physically injecting $CD8^+$ T cells into the bone marrow cavity.

6.2 Testing the effects of physical $CD8^+$ T cell emplacement within the BM upon accumulation and memory phenotype

Experiments with haematopoietic stem cells have demonstrated the utility of intra-bone marrow injection (IB) in enhancing levels of engraftment in non-obese diabetic/ severe combined immunodeficient (NOD/ SCID) mice compared with iv injection (495). This is a straightforward technique with considerable advantages for development. However, as we have seen in the previous chapter, transient enhancement of BM homing does not substantially alter memory phenotype. As we directly observed, dynamic T cell motion is involves rapid movement to and from the BM, so initial physical emplacement may not replicate longer-term interactions with cellular and soluble niche-related factors.

I attempted to replicate the effect of enhanced bone marrow homing by injecting $CD8^+$ T cells into the murine tibial cavity in a competition assay. 5 x 10^6 MACS-sorted $CD8^+$ T cells from B6 splenocytes were injected intravenously (IV) and IB into anaesthetised B6 mice. Numerical and phenotypic outputs were analysed 1 and 2 weeks post injection. Congenic markers were used to distinguish IV and IB cells.

Numerical accumulation

At day 7, the median ratio of IV:IB cells was 3.5:1 in BM, 4.7:1 in spleen and 4.1:1 in LN. At day 14 this pattern was unchanged, such that median BM ratio was 6.2:1, spleen 7.5:1, LN 5.5:1, n=4, data from 2 independent experiments.

Memory phenotype and proliferation

The proportion of T^{CM} phenotype cells was similar for both groups in all organs at both time points (day 7 BM median IB vs IV T^{CM} frequency was 33.0% vs 34.0%, spleen 54.3% vs 55.1%, LN 52.3% vs 55.4%). At day 14, BM T^{CM} frequency was 50.0 vs 30.3, spleen 36.9% vs 44.5%, LN 43.5% vs 52.3%.

Similarly, there was no evidence of changes in cytokine receptor expression for IB vs IV at either time point (day 14 BM CD122 MFI 0 vs 130, spleen 0 vs 76, LN 0 vs 29, day 14 BM CD127 MFI 1585 vs 4633, spleen 1001 vs 1256, LN 1022 vs 1037), day 14 BM CD25 MFI 249 vs 102, spleen 0 vs 194, LN 0 vs 115). Finally, proliferation was similar for IB vs IV (day 14 BM EdU MFI 2909 vs 3172, spleen 2508 vs 2710, LN 2408 vs 2765).

Summary

These findings suggest that physical placement of un-manipulated $CD8^+$ T cells within normal bone marrow does not replicate the T^{CXCR4} phenotype or numerical BM advantage.

The numerical advantage seen for IV cells suggests that the physical constraints of the tibia meant a lower number of cells were successfully injected into the bone marrow cavity rather than surrounding tissue, resulting in excessive loss of the IB cells before they could gain access to survival factors. Given the lack of any advantage seen and technical difficulties with

successful injection into the cavity I then pursued a different approach to enhance BM homing.

6.3 Testing the effects of pharmacological modification of DPPIV expression on BM homing of $CD8^+$ T cells

A number of groups have investigated regulation of haematopoietic stem cell trafficking to the bone marrow niche. One suggested approach to enhance cord blood $CD34^+$ migration in response to CXCL12 is to inhibit CD26/dipeptidylpeptidase IV (DPPIV) (91). CD26 is a membrane-bound extracellular peptidase expressed on the surface of $CD34^+$ haematopoietic cells and activated T lymphocytes. CD26 has been shown to cleave the first two amino acids from certain chemokines, in particular CXCL12, owing to its N-terminal structure (496). Truncated CXCL12, known as CXCL12 (3-68), is unable to induce chemotaxis of haematopoietic stem cells but retains the ability to bind the CXCR4 receptor, acting as an antagonist to normal CXCL12 (91). Treatment of haematopoietic stem cells with DPPIV inhibitors such as Diprotin A (DipA) enhances migration in response to CXCL12.

DPPIV inhibitors have been extensively studied in large clinical trials without safety concerns and are approved for type II diabetes treatment. DPPIV inhibition is an attractive pharmacological option to preserve CXCL12 in its active form and potentially enhance lymphocyte homing to the CXCL12 bone

marrow niche. CD26 peptidase activity is depleted within 15 minutes of DipA treatment and although recovery begins by 4 hours, we postulated that initial preferential access to the BM niche might enhance accumulation and memory formation (92).

In addition to avoiding regulatory issues involved in genetic modification of therapeutic T cells, we could potentially avoid the activation steps required in current retroviral transduction protocols. Transduction unavoidably prolongs time spent in culture, involves use of expensive reagents (to good manufacturing practice (GMP)- grade standards in the clinical setting), and cause some cell loss, differentiation and phenotypic change such as reduction in CD27 expression (497). In a setting where the aim is to preserve a less differentiated subset, this may be deleterious to long-term persistence and stem-like qualities (6, 498). Although many safety issues have been addressed and mature T cells appear to be resistant to tumourigenicity, given the early issues with gene therapy trials, there are still safety concerns surrounding preferential insertion of retroviral sequences into tumour-promoting regions of the genome and a limited truly long-term experience with engineered T cells undergoing extensive clonal proliferation (499, 500).

I therefore hypothesised that exposure of non-activated T cells to DPPIV inhibitors would preserve higher proportions of less differentiated/stem-like T cell memory phenotypes. In addition, the somewhat rudimentary approach of intrabone injection detailed above only deposits T cells within the BM cavity and not a specialized niche, without altering trafficking behaviour. DPPIV inhibition aims to enhance homing specifically to a $CXCL12^{+}$ niche, and is hence more likely to replicate the T^{CXCR4} memory phenotype.

I examined the potential of this approach utilizing polyclonal $CD8^+$ T cells, MACS-sorted from B6 splenocytes and treated with 5 mM DipA for 15 minutes at 37 ºC as per published protocols. These cells were mixed in a 1:1 ratio with congenically distinct PBS-treated $CD8^+$ T cells and injected intravenously into *Rag1ko* mice. The numerical accumulation and phenotype were assessed at days 1 and 7. At day 0, DipA and PBS-treated cells were similar in terms of T^N proportions (79.0% vs 79.1%) and cytokine receptor expression (CD122 MFI 348 vs 365, CD127 MFI 1442 vs 1384).

Numerical accumulation

At day 1, the median ratio of DipA: PBS-treated cells in BM, spleen and LN was 1.1:1, 1:1 and 1.1:1 respectively. Median absolute numbers of cells were 2834 and 2538 in BM, 16,321 vs 14,663 in spleen and 1619 vs 1769 in LN. At day 7 median ratios were similar (BM and spleen 0.9:1, LN 1.2:1). Cell numbers remained similar at day 7 in all organs for DipA and PBS respectively (BM 1038 vs 1143, spleen 14,666 vs 13,153 and LN 10,660 vs 11,202) (Figure 6-1A). Between days 1 and 7, the numbers of BM cells declined for both DipA and PBS (2834 to 1038 and 2538 to 1143, 2 tailed paired t-test, p=0.07 & 0.19), remained similar in the spleen (16,321 to 14,666 and 14,663 to 13,153) and increased in LN (1619 to 10,660 and 1769 to 11,202, 2 tailed paired t-test, p=0.07 & 0.09).

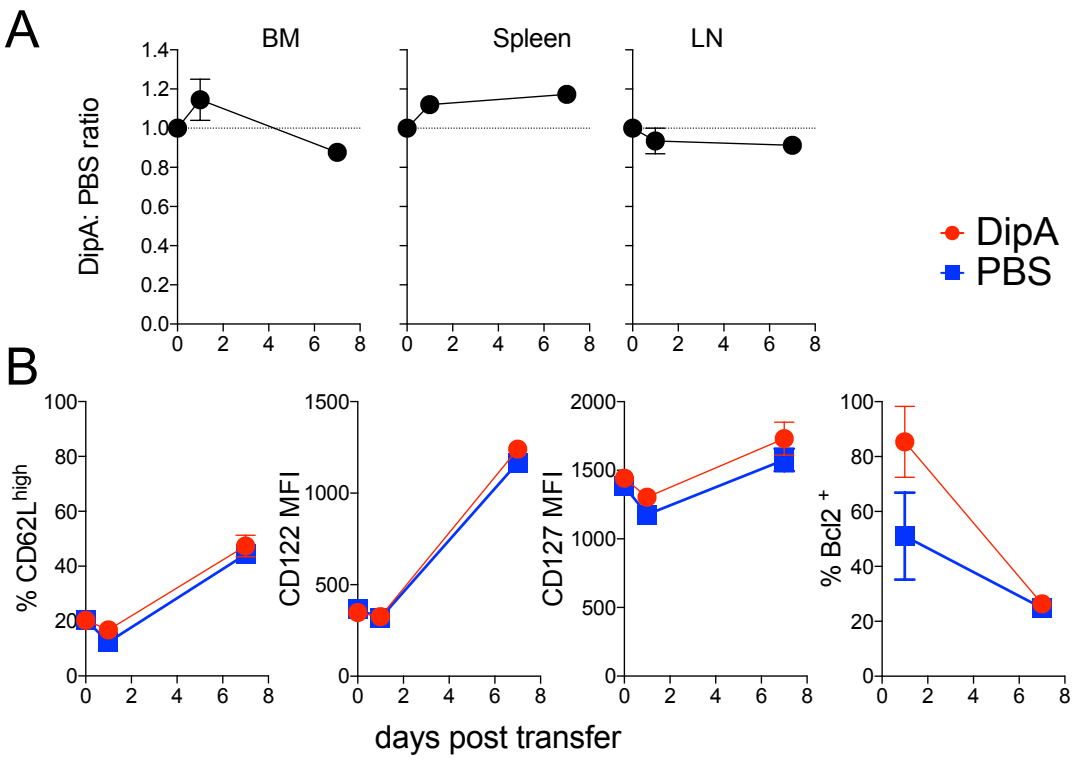

Figure 6-1 Diprotin A treatment does not alter T cell accumulation or memory phenotype.
(A) Ratio of Diprotin A: PBS-treated $CD8^+$ T cells in BM, spleen and LN over time post injection. (B) BM phenotype of Diprotin A-treated (red) and PBS-treated (blue) cells. Data derived from one experiment, n=5.

Memory phenotype and cytokine receptor expression

At day 1, the majority of cells expressed T^N markers in all organs for DipA vs PBS-treated cells (BM $\%T^{CM}$ 16.8% vs 12.4%, spleen 19.5% vs 14.4%, LN 9.4% vs 8.1%). At day 7 both groups possessed a similar proportion of $CD62L^+CD44^+$ T^{CM}-phenotype cells (BM 44.0% vs 45.7%, spleen 66.8% vs 57.9%, LN 53.8% vs 48.1%), (Figure 6-1B).

Receptors for cytokines IL-2, IL-7 and IL-15 were expressed to a similar level on DipA and PBS-treated cells at day 1 (day 1 median BM CD122 MFI 325 vs 318, spleen 353 vs 212 and LN day 1 226 vs 109, median CD127 MFI BM

1304 vs 1177, spleen 923 vs 703, LN 740 vs 545, median CD25 MFI BM -166 vs -129, spleen -121 vs -97, LN -224 vs -166). Median CD132 MFI BM 1366 vs 1016, spleen 3308 vs 2749, LN 10724 vs 11186, 2 tailed paired t-test, p=0.01, 0.02 and ns respectively).

At day 7 CD122 BM and spleen expression were similar (median BM MFI 1225 vs 1154, spleen 743 vs 866) and slightly higher expression was seen in DipA cells for LN (755 vs 590, p=0.01). CD127 expression was also similar in BM and slightly increased in spleen and LN (median BM MFI 1738 vs 1535, spleen 1474 vs 1274, LN 1209 vs 992, p=ns, <0.01 and <0.01 respectively), (Figure 6-1B).

Day 7 DipA CD132 expression was higher in all organs (BM 984 vs 534, spleen 1515 vs 1141, LN 2076 vs 1188, p=0.04, 0.04 and <0.01) and reciprocally CD25 expression was slightly lower for DipA cells at day 7 in spleen and LN (BM 67.9 vs 98.7, spleen 81.9 vs 116, LN 88.4 vs 145, p=ns, 0.03 and 0.005 respectively).

Survival and Proliferation

At day 1 BCL2 %positivity was higher in DipA cells vs PBS (median BM 85.4 vs 51.1, spleen 95.4 vs 75.0, LN 98.3 vs 70.2, p=0.05, 0.03 and 0.07), similarly for BCL2 MFI (median BM MFI DipA 2071 vs PBS 752, spleen 1883 vs 1682, LN 3086 vs 1664, p=0.08, 0.04 and 0.03) however this advantage persisted only in LN at day 7 (BM %positive 27.4 vs 25.0, spleen 44.2 vs 56.4, LN 49.6 vs 23.2, p=ns, 0.02 and <0.01), (Figure 6-1B).

Proliferation as measured by Ki67 staining was higher in all organs for DipA at the early time point (median BM Ki67 MFI 334 vs -89, spleen 265 vs 33, LN 536 vs 95, $p<0.01$, 0.02 and 0.02) however at day 7 the pattern had changed such that proliferation was higher for PBS-treated cells in spleen and equivalent in other organs (BM Ki67 MFI 234 vs 269, spleen 457 vs 945, LN 1188 vs 1246, p=ns, 0.01 and ns).

CXCR4 expression level

CXCR4 expression was similar between DipA and PBS groups in all organs at all time points (median day 1 BM MFI 99 vs 64, spleen 97 vs -14, LN 82 vs 68 and day 7 255 vs 209, 205 vs 267, 267 vs 207, p=ns for all).

Summary

DipA-treated cells showed no enhancement in homing towards the BM niche or differential tendency to form a central memory phenotype in comparison to PBS-treated cells. Small increases were seen in CD127 and CD132 at the later time point in spleen and LN, coupled with a reduction in CD25 expression in DipA cells. BCL2 and Ki67 expression was higher for DipA-exposed cells in all organs at the early time point but by day 7 the only advantage was for BCL2 in the LN.

6.4 Testing the effects of in vivo CXCR4 blockade on primary vaccination response

Thus far physical or pharmaceutical methods have not enhanced either BM homing or memory T cell phenotype. I hypothesised that if CXCR4 over-expression enabled preferential homing to a BM niche enabling memory precursor formation in the context of antigen, blockade of physiological CXCR4 expression on naïve $CD8^+$ T cells during antigen priming would result in reduced BM homing and polarization towards a more differentiated effector phenotype. If this hypothesis was proven, this would suggest physiological levels of CXCR4 on naïve-phenotype T cells are important for BM homing and memory phenotype generation. This in turn would support further exploration of methods designed to improve homing to a $CXCL12^+$ BM microenvironment.

If no evidence could be found to support this hypothesis, this might suggest that physiological CXCR4 levels on naïve non-activated T cells are not sufficient to confer BM homing and are not relevant for memory precursor differentiation in an antigen-dependent setting. This in turn suggests that pharmacological approaches to leverage physiological T cell expression of CXCR4 by preserving intact CXCL12 are less likely to succeed given the likelihood of out-competition by other cells expressing higher levels of CXCR4, and that approaches aimed at increasing basal T cell CXCR4 expression are more likely to be successful.

In the following experiments I used a similar vaccination model as described above to examine the effects of CXCR4 blockade upon memory phenotype and bone marrow accumulation, which differed only in that naïve OT-I $CD8^+$ T

cells were injected into *Rag1ko* mice at day 0 without prior activation or transduction. Mice received either the CXCR4 antagonist Plerixafor or PBS daily from Day -1 to Day 8. The time period for blockade was chosen based upon the antigen-dependent nature of the T^{CXCR4} memory phenotype. I hypothesised that CXCR4 over-expression is particularly important at the time of initial antigen exposure, and hence that blockade during this period would be most relevant to alter phenotype. This supposition is based upon evidence that primary antigen exposure results in distinct T cell fates (189, 191). If we saw differences in effector phenotype polarization post initial vaccination that were then lost upon boost vaccination in the absence of any further CXCR4 blockade, this would suggest the need for continued CXCR4 blockade to prevent BM homing, and therefore perhaps an ongoing influence of homeostatic cytokine exposure.

In addition Plerixafor has low oral bioavailability (501) and daily injections for the duration of the experiment would require justification based upon initial results. Other approaches to inhibit G-protein mediated signalling, for example using Pertussis toxin, would have multiple off-target effects. All mice received primary and boost vaccinations with SIINFEKL peptide on days 1 and 29, and phenotype was assessed as before (Figure 6-2).

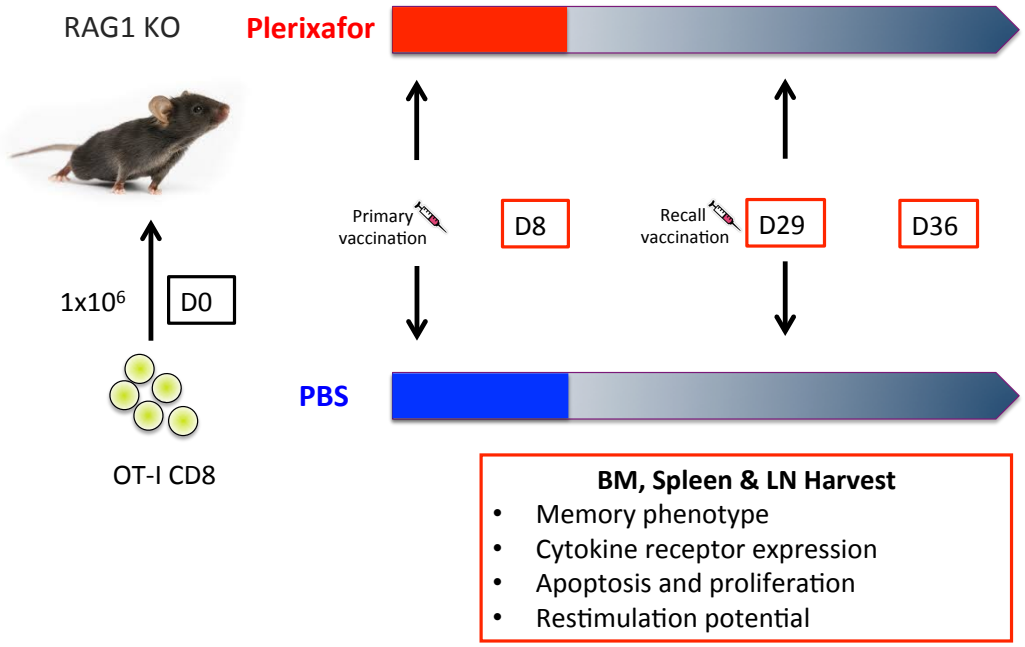

Figure 6-2 CXCR4 blockade vaccination model
Naïve OT-I $CD8^+$ T cells were injected into *Rag1ko* mice receiving daily ip PBS or Plerixafor injections from days -1 to day 8. Organ accumulation and memory response were assessed at days 8, 29 and 36 post SIINFEKL-IFA vaccination on days 1 and 29.

Numerical accumulation

At day 8, 1 week post primary vaccination, the median absolute numbers of cells in Plerixafor-treated animals were numerically lower in BM and spleen compared to PBS-treated animals (BM 19,957 vs 40,817 and spleen 164,365 vs 250,613, p=ns for both, Mann-Whitney test) and higher in LN (56,091 vs 37,431), (Figure 6-3). The BM/LN ratio was similar between Plerixafor and PBS-treated animals (0.5 vs 0.4).

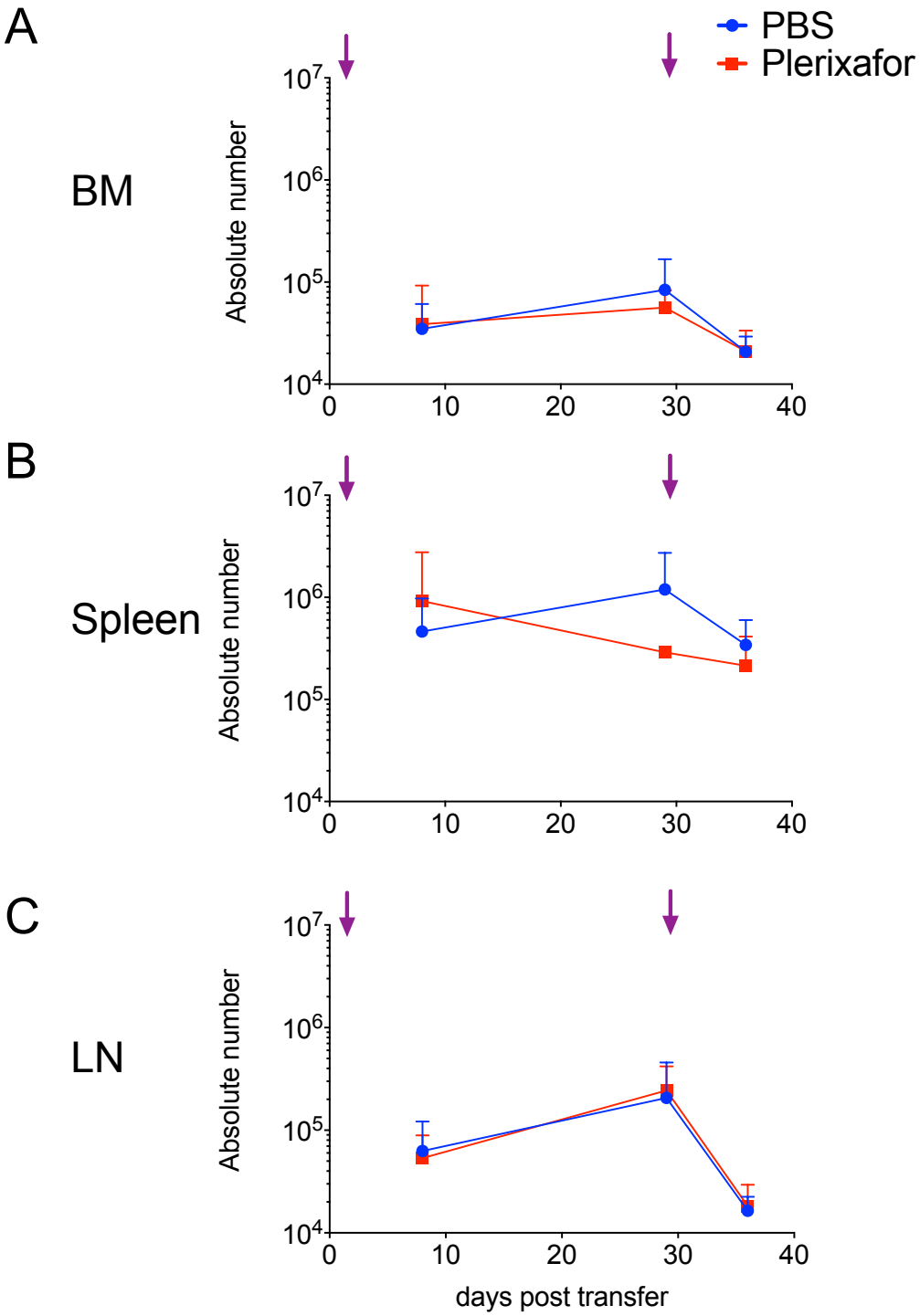

Figure 6-3 CXCR4 blockade organ accumulation data.
(A-C) Absolute cell number kinetics for Plerixafor (red) and PBS-treated mice (blue) over time in bone marrow, spleen and lymph node, n=24, data pooled from 3 independent experiments. Purple arrows indicate time of vaccination, error bars denote SD.

Memory phenotype

At day 0, a median of 86% OT-I $CD8^+$ T cells were classed as T^{naive} ($CD44^-$ $CD62L^+$). At day 8 in vaccinated BM, 23% of Plerixafor-treated and 27% of PBS-treated expressed T^{CM} markers (Mann-Whitney test, p=ns). T^{CM} proportion was similarly unchanged in spleen (28% vs 25%, p=ns) and LN (75% vs 73%, p=ns), (Figure 6-4).

Cytokine receptors, survival and proliferation

There were no significant differences in cytokine receptor expression between vaccinated Plerixafor and PBS-treated animals (BM CD122 MFI 1014 vs 1102, spleen 1085 vs 1076, LN 1223 vs 1294, CD127 MFI 581 vs 562, 585 vs 692, 1271 vs 1220, CD25 MFI 138 vs 256, 163 vs 214, 103 vs 176, CD132 751 vs 697, 945 vs 851, 1302 vs 1071, Mann-Whitney test p=ns for all), (Figure 6-4).

Similarly, there were no differences in survival or proliferation between the 2 groups (BM BCL2 MFI 409 vs 318, spleen 498 vs 454, LN 673 vs 727, BM EdU MFI 1191 vs 1377, spleen 1087 vs 1974, LN 810 vs 983, Mann-Whitney test p=ns for all), (Figure 6-4).

Function

Finally, at the early time point Plerixafor did not alter cytokine secretion upon splenocyte restimulation (IFN-γ MFI Plerixafor 417 vs PBS 479%, TNF-α MFI 410 vs 395 and IL-2 MFI 29.6 vs 17.4, p=ns for all), (Figure 6-4).

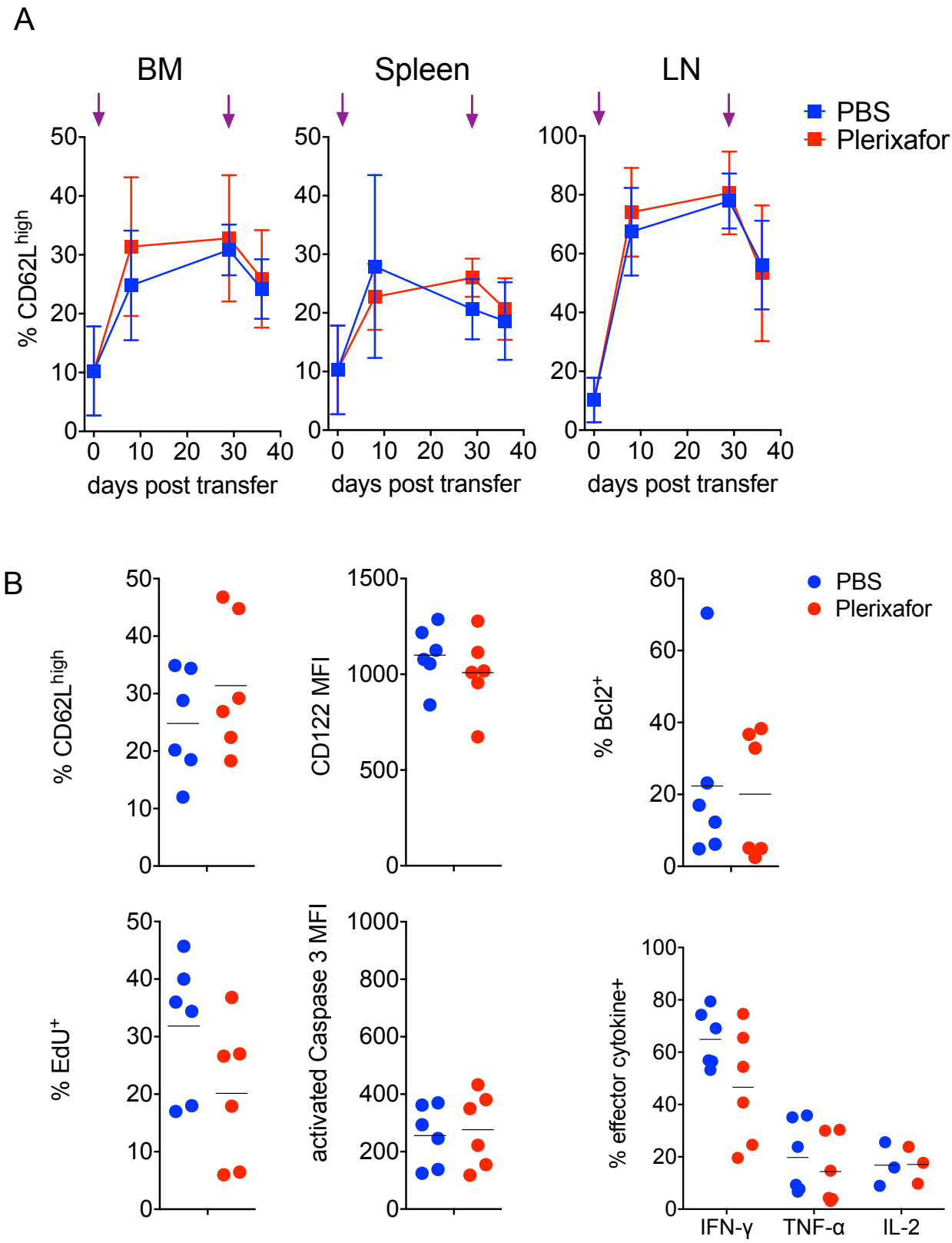

Figure 6-4 In vivo Plerixafor treatment does not alter CD8$^+$ T cell memory phenotype post vaccination.
(A) Summary T cell CD62L^{high} frequency over time in BM, spleen and LN for PBS (blue) and Plerixafor-treated (red) mice. Purple arrows

indicate time of vaccination. Error bars denote SD, n=24, data pooled from 3 independent experiments. (B) Summary data for BM CD62L^{high} frequency, CD122 MFI, % BCL2+, %EdU^{+}, caspase 3 MFI, and cytokine release post splenocyte restimulation in PBS and Plerixafor-treated mice post initial vaccination at day 8, n=12, data pooled from 3 independent experiments.

6.5 Testing the effects of boost vaccination following initial CXCR4 blockade

Numerical accumulation

At day 36, the median absolute numbers of cells in Plerixafor-treated animals were similar in all organs compared to PBS-treated animals (BM 20,402 vs 21,288, spleen 146,268 vs 330,752, LN 19,044 vs 18488, p=ns for all, Mann-Whitney test), (Figure 6-3). BM/LN ratio remained similar between Plerixafor and PBS-treated animals (1.3 vs 1.4).

Memory phenotype

At day 36 in the BM, 27% of Plerixafor-treated and 26% of PBS-treated expressed T^{CM} markers (Mann-Whitney test, p=ns). T^{CM} proportion was also similar in spleen (20% vs 20%, p=ns) and LN (55% vs 62%, p=ns).

Cytokine receptors, survival and proliferation

There were no significant differences in cytokine receptor expression between Plerixafor and PBS-treated animals (BM CD122 MFI 1130 vs 969, spleen 620 vs 615, LN 966 vs 1071, CD127 MFI 442 vs 525, 411 vs 325, 1077 vs 1393, CD25 MFI 175 vs 121, 130 vs 105, 195 vs 125, CD132 800 vs 809, 649 vs 639, 1108 vs 1089, Mann-Whitney test p=ns for all). Similarly, survival and proliferation were similar in all organs (BM BCL2 MFI 3234 vs 3193, spleen 1705 vs 2182, LN 4905 vs 4515, BM EdU MFI 2673 vs 2688, spleen 1989 vs 1948, LN 1946 vs 1959, p=ns for all).

Function

Upon restimulation post boost vaccination, there were no functional differences apparent between Plerixafor and PBS-treated mice (IFN-γ MFI 417 vs 479, TNF-α MFI 410 vs 395, IL-2 MFI 30 vs 17, p=ns for all).

Summary

Short term Plerixafor treatment did not induce any changes in numerical accumulation or memory $CD8^+$ T cell phenotype, either during the initial period of CXCR4 blockade post primary antigen exposure or following boost vaccination.

6.6 Discussion

Intra-bone injection & DPPIV inhibition

Neither physical intrabone injection, ex vivo inhibition of DPPIV or in vivo CXCR4 antagonism were able to substantially alter T cell BM accumulation or modify memory phenotype. The lack of effect of direct BM emplacement of $CD8^+$ T cells is perhaps unsurprising given that T cell migration into the BM is a complex multi-step process requiring sequential activation and interaction of a series of molecules including selectins and integrins. In the absence of directed migration, the speed of blood flow is likely to rapidly re-distribute injected T cells before substantial migration into the BM parenchyma occurs. Furthermore, judging from the rapid transient homeostatic behaviour of imaged T cells within the parenchyma, injected BM T cells are unlikely to be preferentially retained in the absence of altered signalling at a molecular level. In vivo data demonstrating that memory T cells only transiently retain some traits of previous stimulation following exit from the BM support this conclusion (502).

The lack of effect of DPPIV inhibition on cell number or phenotype is unexpected. The impressive effects of CD26 inhibition seen in mouse and human haematopoietic stem cells in terms of BM engraftment were not seen in $CD8^+$ T lymphocytes following ex vivo inhibition. This issue might stem from differential CD26 expression on T cells. I used bead-sorted non-activated naïve $CD8^+$ T cells, which express lower levels of CD26 than activated and memory T cells and might therefore exhibit less pronounced responses (503).

It is also possible that the short exposure to Diprotin A was insufficient to inhibit CD26. One way to test for effective inhibition would be a DPPIV Protease Assay, combining a luminogenic DPPIV substrate (Gly-Pro-aminoluciferin) and a luciferase enzyme. This generates a luminescent signal to quantitate remaining DPPIV activity present with greater sensitivity and accuracy than fluorescence-based assays (96). We could further test the effect on CXCL12-induced T cell migration in vitro using Transwell migration assays following Diprotin A exposure.

The more likely explanation, given the successful use of this protocol to modulate stem cell homing, is that DPPIV was successfully inhibited by treatment but as CD26 recovery begins within 4 hours of treatment (92), this is insufficient time to allow the treated T cells to gain access to BM-based factors mediating the memory phenotype. BM homing may have been only transiently enhanced and migration patterns then return to normal. This would be in keeping with results from the intrabone injections, and the lack of effect of transient expression with the inducible vector.

It may also be relevant that Diprotin A treatment only enhances CXCL12-induced migration two-fold in ex vivo C57BL/6 Sca-1^+ Lin^- BM cells, and in vivo homing of HSCs on a short-term basis (92). Work demonstrating enhanced lymphocyte trafficking in mice and enhanced cord blood transplant engraftment in patients has employed the DPPIV inhibitor sitagliptin (Januvia), which has been approved by the European Medicines Agency (EMA) (94, 96). Other BM transplantation models failed to confirm enhancement of engraftment by Diprotin A or sitagliptin despite proven DPPIV inhibition (504). Our data from Chapter 4 suggest that high-level CXCR4 expression creates a

very distinctive phenotype that is not replicated by lower level expression. A longer duration of signalling and/or more potent effects may be required to mediate durable change in phenotype and fate. Further experiments should therefore examine the effects of longer term and more potent in vivo inhibition on BM homing, memory responses and tumour infiltration.

In vivo CXCR4 antagonism

The effect of once daily Plerixafor in animals receiving predominantly naïve-phenotype $CD8^+$ T cells is negligible. This effect is perhaps slightly surprising given the dramatic effects of CXCR4 over-expression, and the effects of Plerixafor in altering haematopoietic stem cell homing. Two possible reasons present themselves. Firstly, relatively low CXCR4 expression on unmanipulated $CD8^+$ T cells suggests lower likelihood of success in accessing CXCL12-abundant reticular cell niches, particularly in the context of competition from $CXCR4^{high}$ haematopoietic stem cells. Naïve T cells home preferentially to lymphoid organs expressing selectin ligands such as lymph nodes and hence Plerixafor-treated mice may simply undergo antigen-dependent priming in lymph nodes to form memory precursors. This phenomenon may be more pronounced in the context of abundant homeostatic cytokines in immunodeficient mice. Secondly, Plerixafor has a short half-life of 4-5 hours (505), meaning that the impact of once daily administration may be insufficient to ensure T cells do not interact with BM microenvironments mediating a memory phenotype. Once drug levels have diminished, cells may home to the BM niche and gain sufficient access to

cytokines and other mediators to prevent alterations in phenotype. Evidence that Cre-mediated deletion of CXCR4 in $CD8^+$ T cells results in impaired BM homing of both T^N and T^{CM} with impacts upon the size of the T^{CM} pool in secondary lymphoid organs suggests that basal CXCR4 signalling is important for homeostatic self-renewal, and therefore that the second explanation is more likely (305).

Interestingly CXCR4-deficient T cells can undergo normal rechallenge self-renewal in the presence of antigen, producing equivalent proportions and numbers of T^{CM} phenotype cells to CXCR4-sufficient $CD8^+$ T cells (305). This suggests that differing mechanisms for homeostatic and antigen-dependent self-renewal may also explain our experimental findings. In the context of lack of CXCR4-mediated BM homing, CXCR4-independent T^{CM} formation and maintenance induced by antigen may occur predominantly in spleen and LNs, resulting in sufficient production of the full range of memory phenotypes. Thus, differences in memory phenotype would then not be apparent unless extended duration constant inhibition of CXCR4 reveals impaired homeostatic maintenance of memory precursors, and terminal differentiation in response to repetitive antigenic stimulation.

It would be interesting to perform further experiments with twice daily Plerixafor in both the antigen-dependent and independent setting, to confirm effects upon BM homing. Further experiments using longer term CXCR4 inhibition or stronger antagonists, and comprehensively profiling markers of senescence and differentiation such as T-bet, Eomes and PD-1 after multiple

antigenic exposures would be important to test the effects of basal CXCR4 expression in T cells. This is potentially important if one wished to pursue a strategy of pharmacological enhancement of BM homing in $CD8^+$ T cells.

7 Discussion

7.1 Putative mechanisms underlying the early memory T^{CXCR4} phenotype

The experimental results obtained in Chapter 4 suggest that constitutive CXCR4 over-expression enhances BM accumulation and polarises $CD8^+$ T cells to a $BCL2^{High}$ IL-15Rβ^{High} polyfunctional central memory phenotype that is dependent upon IL-15Rα signalling. These data are consistent with transcriptional analyses confirming expression of a memory precursor-like signature (222, 506). This finding could be due to several possible mechanisms.

Firstly, preferential localisation to a CXCL12-secreting IL-15Rα^+ stromal niche (**IL-15Rα^+ niche mechanism**) could enhance preferential retention and survival of a $CD62L^+CD122^{High}$ memory phenotype. CD122 expression is required for responsiveness to trans-presented IL-15 through formation of the heterotrimeric receptor (507). We were not able to obtain direct imaging evidence for privileged interaction with this cellular niche, perhaps due to limitations of the antibodies available. The finding of IL-15 dependence for the phenotype could be interpreted as further evidence in support of this hypothesis.

Secondly, central memory polarization could relate to alterations in the nature of T cell-APC interactions, mediated by enhanced CXCR4 expression (**altered priming mechanism**). CXCR4 is known to be recruited into the immunological synapse, hence altered signalling as well as migration patterns will influence the quality and duration of synapse formation. Strength of signal and competition for antigen are known to influence asymmetric T cell division and unequal partitioning of cell molecular regulators, and hence the extent of differentiation and memory fate (190, 191, 484). Recent findings have suggested that curtailing T cell activation can generate T stem memory cells with low effector-associated genes and considerable self-renewal potential in response to IL-15 (508). Once formed, central memory T cells also preferentially rely upon IL-15 for upregulation of BCL2 and T^{CM} survival is markedly impaired in *IL-15Rako* mice. The few remaining T^{CXCR4} cells in our *IL-15Rako* experiments predominantly express an effector memory/effector phenotype. In the absence of IL-15 signalling, it is likely that rely upon the same survival pathways as $T^{Control}$ cells, predominantly IL-7. This would explain the T^{CXCR4} requirement for IL-15 signalling. Although the complexities of dynamic long-term interactions with multiple cellular components with a three-dimensional niche cannot be recapitulated in a short-term tissue culture environment, in vitro antigen activation demonstrated no differences between T^{CXCR4} and $T^{Control}$, excluding a direct costimulatory effect of CXCR4 upon T^{CXCR4} phenotype.

We saw distinct differences in movement when imaging steady state T^{CXCR4} and $T^{Control}$ that offer clues as to how phenotype may be shaped. T^{CXCR4} could be divided into two distinct populations: arrested cells moved very slowly, if at

all, whilst migratory cells displayed significantly greater speeds. No such distinction could be made between $T^{Control}$ cells. In addition, T^{CXCR4} tracks were significantly less straight, suggesting diversion in response to chemokine cues. One might hypothesize that T^{CXCR4} interactions with APCs in an inflammatory setting would also be more disrupted by chemokine cues than T^{CXCR4}, supporting the altered priming mechanism. However, despite profound differences in memory phenotype between $T^{CXCR4\ HIGH}$ and $T^{CXCR4\ LOW}$, we did not observe differences in speed or arrest coefficient. This suggests that dynamic behaviour does not reflect phenotype, but requires further study.

A third possible mechanism is that the enhanced memory phenotype could relate to preferential access to IL-15 throughout the BM parenchyma, not just a specific microenvironment or niche. Enhanced numbers of T^{CXCR4} in the BM might lead to more numerous stochastic interactions with either stromal or haematopoietic sources of IL-15. It is also possible that soluble IL-15/IL-15Rα complexes (sIL-15 complexes), as opposed to trans-presented IL-15, contribute towards enhanced T cell responses, as no models yet exist to distinguish these effects (509). This mechanism is less likely because T^{CXCR4} demonstrate a preferential T^{CM} phenotype in comparison to $T^{Control}$ within the BM itself.

A final possibility is that T^{CXCR4} undergo lower levels of antigen exposure due to a quantitative lack of DC-mediated antigen presentation in the BM as opposed to the LN where $T^{Control}$ might undergo activation. This is also less likely because of the preferential T^{CXCR4} T^{CM} phenotype in comparison to $T^{Control}$ within the BM itself. Indeed, our method has been used to visualise

dendritic cells triggering recall responses through interactions with central memory T cells within the BM itself (510).

Prolonged duration imaging experiments in the context of antigen-exposure will be important to investigate changes in T^{CXCR4} cell behaviour in inflammatory and tumour-specific models, and the importance of antigen-presenting cell interactions. Using homeostatic cytokine (311) and stromal reporter mice (281, 282) would enable precise identification of the mechanism underlying the T^{CXCR4} phenotype, and potentially provide important and generalizable new insights into enhancing formation of more potent memory T cell phenotypes.

Both the IL-15Rα^{+} niche and the altered priming mechanisms are consistent with enhanced bone marrow persistence and absolute numbers of T^{CXCR4}. The superior persistence in secondary lymphoid organs shown in both homeostatic and inflammatory settings can be explained by increased numbers of T cells homing to the bone marrow. This could lead to more numerous stochastic interactions with either stromal or haematopoietic sources of IL-15, mediating enhanced survival and greater cell number, consistent with the superior expansion potential of central memory T cells. A proportion of BM-derived T cells then recirculate to other organs, enhancing persistence in all organs sampled. Direct observation of close clustering between bone marrow T^{CXCR4}, in contrast to isolated $T^{Control}$, further suggests that these cells might provide survival factors for each other, for example by paracrine IL-2 secretion in an inflammatory situation, as seen in our ex vivo antigen restimulation data.

A second question following on from this raised by our observations is the specific cellular source of IL-15, which may be important for generation of the distinctive phenotype. Dendritic cell-derived IL-15Rα has been suggested to preferentially support central memory $CD8^+$ T cells while macrophage-derived IL-15Rα supports both T^{CM} and T^{EM}. Although the mechanism remains unclear, it may relate to the strength of TCR and cytokine signalling (484, 511, 512). This is relevant because of the potential for combination immunotherapy. Administration of an agonist anti-CD40 antibody increases expression of IL15Ra on $CD11^+$ DCs and prolongs survival in tumour-bearing models in combination with IL-15 in additive benefit to either agent alone (513). If T^{CXCR4} are accessing a dendritic cell-IL-15Rα niche this opens up the possibilities of engineering tumour-specific responses based upon tunable cytokine and small molecule delivery (514). IL-15 functions as a tissue distress signal and reduces the TCR activation threshold, hence intensification of IL-15 generation and costimulatory signalling during trans-presentation might provide an amplification loop to mediate tissue and tumour destruction, particularly in situations of high IL-15 bioavailability, such as BM-derived malignancies (515).

Transcriptional profiling of T^{CXCR4} has shown upregulation of several IL-15-associated genes including *Cpt1a* (506). IL-15 promotes mitochondrial biogenesis and spare respiratory capacity in memory T cells by enhanced expression of carnitine palmitoyl transferase, the product of *Cpt1a*, required to initiate fatty acid oxidation in the mitochondria (516). Furthermore, gene set enrichment analysis (GSEA) of T^{CXCR4} confirms enrichment for a memory stem cell-like signature that is also induced in human T cells modified to

undergo constitutive IL-15-IL-15Ra signalling (506, 517). The suggestion that T^{CXCR4} display a different metabolic profile, preferentially employing oxidative phosphorylation over glycolysis, would also be consistent with a stem cell-like T memory profile, and open the door to combinatorial enhancement of T cell potency, perhaps by pharmaceutical modification of T cell metabolism (518). Studies investigating memory T cell generation have highlighted the importance of an appropriate balance between effector and memory cells for anti-tumour potency, and further data is needed on whether combinatorial strategies aimed at modifying phenotype are synergistic or counter-productive (491). Despite central memory polarization, both $CD62L^{High}$ and $CD62L^{Low}$ T^{CXCR4} accumulate to a greater extent than $T^{Control}$ in secondary lymphoid organs in our anti-tumour models, highlighting dual benefits of this strategy.

Our data suggest increased accumulation of T^{CXCR4} relates primarily to enhanced cellular survival, as we saw no evidence of enhanced proliferation. Some data suggest that, unlike the situation in the spleen, BM memory T cells are less susceptible to chemotherapy agents such as cyclophosphamide as they do not rely upon proliferation to enhance cell numbers (519). The concept that at least two memory T cells niches exist within the BM has been proposed as a division of labour to explain seemingly contradictory findings of different groups suggesting both cell cycling and quiescence to be prominent features (262).

Our data could be interpreted as supporting the latter concept although given the heterogeneity of the BM stroma these features may be dictated by specific localisation to certain factors, such as IL-15 in the case of T^{CXCR4}. It may be that T^{CXCR4} are more appropriately described as accessing a cell-survival

niche, as proliferation in either the antigen-independent or dependent setting is generally similar to $T^{Control}$ whilst anti-apoptotic molecule expression and accumulation are enhanced. In addition, our imaging data highlight key differences in T cell behaviour, suggesting division into at least two subsets on the basis of dynamic motion.

Imaging single cell interactions with IL-15Rα^{+} cells may shed light on the complex picture of T cell fate and offers the prospect of finally resolving this issue. It will be interesting to see if the anatomical localisation of survival and proliferative T cell niches parallels the association of HSCs with LepR^{+} CXCL12^{High} perisinusoidal niches, or if function and form vary flexibly and are more appropriately defined by molecular signalling (520-522). In our model we did not observe the CD44^{low} phenotype of putative T^{SCM} but given that quiescent memory T cells can be mobilised from the BM by Plerixafor (264), the development of more potent CXCR4 inhibitors may also offer the opportunity to investigate the physiological presence and anatomical localisation of quiescent memory stem or resident memory T cells (523, 524).

In terms of further exploration of this therapeutic strategy, it would be interesting to study the effect of CXCR4 over-expression on therapeutic CD4^{+} T cells. Niche-specific homing of CCR9^{+} CXCR3^{+} CD4^{+} T cells has been shown to be crucial for the success of PD-1 blockade in epithelial cancers (367), and the preferential in vivo expansion of important CD4^{+} subsets such as T_{reg} cells is of enormous potential value in treatment of auto-immunity and transplantation-related toxicity. Cytokine-based therapies to expand pre-existing in vivo populations have not yielded dramatic benefits (525, 526). Given that CD4^{+} T cells may inhabit slightly different BM niches owing to

differential homeostatic cytokine usage, the $CD4^+$ T^{CXCR4} phenotype may be subtly different from their $CD8^+$ counterparts. Memory $CD4^+$ T cells are known to undergo enhanced proliferation in response to IL-15 (527) and imaging data from reporter mice shows considerable overlap between IL-15 and IL-7 expressing $PDGFR\alpha^+$ BM stromal cells likely to represent CXCL12-abundant reticular cells (311, 528), suggesting that detailed analysis of $CD4^+$ T^{CXCR4} would be interesting to support translational work.

Tumour-specific discussion

I would like to conduct further experiments to test the ability of T^{CXCR4} to mediate polyfunctional responses to tumour antigen following repetitive exposure, to assess a full checkpoint profile for evidence of tumour-related exhaustion, and to examine the effects of T^{CXCR4} in known immune evasive settings. The A20 tumour stroma expresses CXCL12, and although T^{CXCR4} homing to the tumour site was not enhanced, heightened CXCR4 expression might enhance the costimulatory effects of CXCL12 upon the TCR, for example upon cytokine secretion. It is therefore important to test these constructs in alternative tumour models, preferably patient-derived xenografts.

7.2 Alternative strategies to enhance BM homing and CXCR4 expression

A pharmacological approach to modify BM homing is more attractive in terms of ease of clinical translation and requires in-depth investigation, which unfortunately time did not allow. The effect of DPPIV inhibition might be more relevant in future experiments exploring the use of ex vivo activation prior to use of Diprotin A, or in vivo vaccination of mice on a sitagliptin-containing diet.

The effect of DPPIV inhibition upon other immune cells needs to be carefully assessed. $CD26^{High}$ $CD4^{+}$ T cells have been proposed as multi-functional cells capable of mediating antitumour regression, as well as activated B cells and DCs (529). Administration of DPPIV inhibitors in vivo may also enhance non-CXCR4 mediated lymphocyte homing. For example, murine melanoma growth and lung metastases are reduced by DPPIV inhibition, due to suppressed truncation of CXCL10, leading to enhanced $CXCR3^{+}$ T cell recruitment into the tumour parenchyma (96). This strategy is enhanced by use of an adjuvant CpG oligodeoxynucelotide to induce CXCL10 formation, which could also be explored in the context of CXCL12 (96).

Unbiased large-scale flow or mass cytometric analysis of a wide range of chemokine receptors may be required to tease apart the precise effects of CD26 inhibition upon T cell trafficking, which may vary dependent upon tumour-specific expression of CD26 and chemokine ligands. Furthermore, mouse DPPIV has significant differences from the human protein, notably a lack of binding to adenosine deaminase, meaning that murine models may not

be predictive of clinical effects (530, 531). DPPIV costimulation has been reported to induce T_{reg} cells with high expression of the LAG-3 coinhibitory protein. In summary, DPPIV inhibition might therefore have unpredictable effects upon immune responses, requiring further study, which might be enhanced by prospective cancer incidence registries for the large numbers of people receiving DPPIV inhibitors worldwide (532).

Finally, a counter-intuitive method to enhance CXCR4 expression is suggested by data revealing that low-dose endogenous glucocorticoids induce IL-7Rα and CXCR4 expression in $CD4^+$ and $CD8^+$ T cells, resulting in diurnal homing to CXCL12 producing cells in spleen and immuno-enhancing memory responses (533). Glucocorticoids and histone deacetylase 5 (HDAC5) inhibition promote CXCR4 expression and homing in HSCs via epigenetic regulation (534, 535). Further work along this line to examine the effects of this form of immune-enhancement could focus on depth and quality of immune responses mediated by T cells in the bone marrow by targeted glucocorticoid signalling or use of other epigenetic modulators. Modulating diurnal variation has the advantage of enhancing physiological mechanisms and therefore less likelihood of off-target side effects. Alteration of T cell migration by enforced homing towards CXCL12 would also be an attractive strategy in autoimmune conditions characterised by aberrant T cell migration (536).

7.3 Identifying the role of T^{CXCR4}: deficiencies in current IL-15-targeting strategies and cellular therapies

Strategies aimed at exploiting the anti-tumour effects of IL-15 are well documented however they suffer from a lack of specific application to the tumour environment and have yet to translate into clinical therapies. Recombinant IL-15 (rIL-15) has a short half-life and requires very high doses for efficacy (537). In addition to its role in enhancing survival and function of immune cells, injection of IL-15 also expands $CD215^+$ myeloid cells and promotes tumour progression (538). Exploiting transpresentation through IL-15/IL-15Rα complexes is more attractive as cell-directed signalling allows for focused delivery to specific cell types, and lower potential for off-target side effects linked to IL-15 such as autoimmunity. The use of fusion protein super agonists such as ALT-803 has the potential to mitigate some of these issues but retains numerous issues including off-target impact and the need for frequent dosing due to a half-life of 8 hours (539). A recent clinical trial of ALT-803 for relapse post-allograft demonstrate the ability to stimulate proliferation of $CD8^+$ T cells, but the low rates of response to single agent therapy highlight the benefits of a cell-specific approach (540). The one-off infusion approach of ACT offers numerous advantages, particularly tumour-targeted efficacy and potentially cost-effectiveness.

Two of the key hurdles in successful clinical translation of adoptive T cell therapy are lack of expansion of sufficient numbers of viable cells and poor in vivo survival. Current ACT protocols predominantly use undefined and

multiply stimulated T cells of effector memory/effector phenotype. These cells are likely to develop functional deficits and exhausted-anergic phenotypes in the usual CAR-T setting of high antigen levels and persistent high-level stimulation. Lack of long-term persistence is strongly associated with frequent relapses, as evinced by rapid recovery of peripheral blood B cells in most protocols (541). The only study to demonstrate persistent aplasia over 26 months used a 4-1BB costimulatory domain (itself a bone marrow-associated 'late-acting' costimulatory agent supplying survival signals) in paediatric patients (542). Given the poor thymic function and immunosenescent T cell phenotype present in the elderly patients likely to be the major recipients of these therapies, there is a pressing need for strategies to maintain potency and early phenotypes in the face of multiple exhaustion-promoting signals (543).

The long-term persistence and expansion of T cells maintained by BM niches in our study suggests that superior long-term capacity for immune surveillance is provided by central memory T cells. This is notably different from the effector memory T cells obtained by many clinical protocols, including IL-12-secreting 3rd generation 'armoured' CAR-T cells, and avoids multiple genetic modifications required to create TCR-deleted (TRAC) CAR-T cells (544-547). Demonstration of long-term persistence is one of the key indicators required for CAR-T cell and other adoptive cell therapies to be able to replace allogeneic HSCT as definitive therapy for poor prognosis haematological malignancies.

It is likely that tumours presenting with a high mutational load will have developed immunosuppressive micro-environments as a counter-measure, hence combination therapies potentially employing CPI in conjunction with adoptive cell therapy may be required to overwhelm tumour resistance. In contrast, those tumours with a low somatic mutation burden have less evolutionary pressure to develop immuno-evasion strategies, and would respond well to single-agent antigen-targeted therapies, as seen for CAR-T cells in B-ALL (<1 mutation/megabase) (542).

The degree of heterogeneity within tumours has been shown to correlate with reduced immune infiltration and lack of response to CPI (411, 548). Further studies are required to establish whether enhancing cell number and modulation of the memory-exhaustion phenotype can overcome this barrier. Data from checkpoint inhibitor trials in melanoma patients suggest that response is a matter of the ratio of T cell invigoration to the magnitude of tumour burden, implying that success is a matter of engineering, but this may not be true for less immunogenic tumours (361).

The impact of spatially distinct tumour microenvironments using a variety of immune evasion strategies therefore needs to be assessed when designing targeted therapies (549, 550). This distinction is particularly important in solid cancers but is also relevant in haematological cancers due to different stromal components of malignant niches in bone marrow, spleen and lymph nodes. The challenges of targeting therapeutic T cells to a variety of cellular niches therefore need to be addressed by longitudinal studies assessing dynamic heterogeneity in four key spheres: the tumour, the immune environment, the stromal environment and the microbiome, which will take some time.

Another concern in the treatment of multiple relapsed-refractory patients is the possiblity of GVHD in the post-allogeneic transplant setting. An increased risk might be expected with the use of 'off the shelf' allogeneic CAR-T cells such as UCART19, with the potential for expansion of T cells retaining their TCR despite genetic disruption (551). We did not see any evidence of increased GVHD in linked work, despite enhanced clinical efficacy (506). The high expansion capacity of T^{CXCR4} suggests that infusing low numbers of oligoclonal T cells might minimise the risk of GVHD associated with large doses of polyclonal T cells (552).

Contrary to previous suggestions, human T^{CM} provide primary tissue-based immunosurveillance, have considerable effector functions and express tissue-homing receptors allowing entry to non-inflamed tissues, suggesting utility in combating immuno-evasion (553). These findings are in line with clinical data suggesting that use of T^{CM}-enriched therapies is safe and effective, and positioning therapies aimed at enhancing bone marrow homing as a potential route to render allogeneic CAR-T protocols more clinically feasible (398). An alternative approach to mitigating the risk of GVHD is to use lymphoid progenitors tolerised by thymic maturation post-allograft, but this is challenging given the thymic atrophy seen in most elderly allograft recipients and the extended period of delayed T cell maturation permits disease relapse particularly in high-risk malignancies (554).

7.4 Limitations of CXCR4 over-expression

The functional attributes of T^{CXCR4} may have unintended drawbacks. Firstly, altered homing to one particular organ may compromise homing to other crucial organs such as the tumour bed or lymph node. Although the BM can act as a location for antigen-dependent priming, evidence that the BM niche can be damaged or altered both by chemoradiotherapy and by malignant cells themselves might limit the ability of BM-homing T cells to traffic to protective niches and develop the memory phenotype. Although we saw enhanced anti-tumour efficacy in our B cell lymphoma model, we were not able to test the efficacy of T^{CXCR4} in models where new data has shown the BM niche to be compromised. For example, acute myeloid leukaemia blasts can suppress BM adipocytes, impairing myeloid maturation, although the fact that lymphocyte levels are normal and lymphoid development is minimally affected suggests that a spatially distinct T^{CXCR4} niche might be unaffected (555).

In contrast, data from my collaborator's lab using a Notch-driven T-acute lymphoblastic leukaemia model suggests that malignant lymphoid clones destroy the osteoblastic BM niche, while retaining perivascular elements. These data highlight the importance of testing ACT in appropriate humanised tumour models in conjunction with clinical data. Interestingly, and in contrast to previous theories, tissue-wide in vivo confocal BM imaging of leukaemia cells at presentation and relapse revealed they did not associate with any particular cellular niche, but retained highly dynamic cellular interactions, with some similarity to the in vivo behavior of our gene-modified T cells (301). Any

therapeutic targeting of these interactions would hence need to target multiple elements of the bone marrow microenvironment, or would need to demonstrate similar abilities in terms of rapid mobility and cellular interactions, as demonstrated by the in vivo imaging behavior of our therapeutic T cells.

One possibility to bear in mind is that these malignant lymphoid cells might compete for the same rate-limiting survival factors such as IL-15, and hence expansion of ACT might be affected, although malignant cells, particularly immature subtypes, use transcriptional deregulation, driven by oncogenes and super-enhancers, via distinct molecular pathways (556). Malignant T-ALL also uses higher expression of CXCR4 than normal T cells to gain access to the BM niche and CXCR4 signalling is known to be required for the survival of T-ALL through interactions with $CXCL12^{+}$ endothelial cells rather than Leptin $Receptor^{+}$ CXCL12-abundant reticular cells (128, 557).

Interestingly, this finding dovetails with our observations regarding endothelial cell expression of CXCL12 and preferential association with T^{CXCR4} (Figure 3-5 and Figure 3-8). VE-$cadherin^{+}$ endothelial cells are known to be the second most abundant source of CXCL12 in BM, but at a magnitude 125-fold lesser than $LepR^{+}$ perivascular cells. Given that $LepR^{+}$ perivascular cells are the major source of CXCL12 in the BM, and that cells mobilise down the chemokine concentration gradient, $CXCL12^{low/intermediate}$ endothelial cells may have unexpected roles in maintenance of T^{CXCR4}. It is possible that our use of a novel dynamic in vivo imaging model with intravenously injected CXCL12 antibody caused preferential binding to endothelial cells ahead of extravascular stromal niches due to diffusion kinetics. An alternative explanation for the imaging evidence of T^{CXCR4} association with endothelial

CXCL12 is fugetaxis from excessively high CXCL12 concentrations surrounding perivascular cells towards $CXCL12^{low/intermediate}$ endothelial cells.

This finding may have bearing when considering use of T cells over-expressing CXCR4 in the context of T-cell malignancies, but may not otherwise be relevant given distinct niches identified for HSCs, myelo-erythroid and lymphoid progenitors. This imaging result suggests that vascular access to the BM is important in addition to other putative perivascular or osteoblastic niches. Clarification is limited by the lack of adequate histological markers. However endothelial cells are not known to be a source of IL-15 nor have they the ability to trans-present via CD215, suggesting that T^{CXCR4} must be accessing an additional IL-15 rich niche. The prime candidate would be IL-15Rα^{+} perivascular stromal cells (311). These data can be reconciled by observation of the dynamic in vivo behaviour of T^{CXCR4}. These cells exhibit a wide range of motility patterns over extended imaging period. Some cells localise to a particular area whilst others range widely, without obvious association with level of CXCR4 expression. The consistency with the T-ALL imaging data suggests that multiple dynamic stochastic interactions are relevant for BM T cells rather than the old paradigm of a fixed niche for each cell-type.

Further work using novel niche models such as the perivascular niche-on-a-chip or three-dimensional biological scaffolds would be helpful to analyse the precise spatial location of BM-homing T cells in terms of the molecular interactions with different tumour models (558, 559). Novel bacterial sortase A-mediated cell labeling could be used across immune synapses to label and

record dynamic in vivo cellular interactions and identify important receptor-ligand interactions mediating immune killing or evasion (560).

A current limitation in gene modifying therapeutic $CD8^+$ T cells is the need to activate and retrovirally transduce naïve cells. Although retroviral transduction with currently used vectors is far safer than historical tools associated with insertional mutagenesis, newer modalities employing clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 (Cas9) and other techniques are potentially far more tractable and easily deployed, and may offer the additional benefit of reducing excessive activation and differentiation. Other recent developments in the field of lentiviral vectors offer the possibility of transducing non-activated cells at extremely high efficiencies (561).

In relation to the risk of insertional mutagenesis, concerns have been expressed over the risks associated with generation of a long-lived stem-like cell with resistance to pro-apoptotic signalling. Adult T-cell leukaemia/lymphoma (ATLL) is a rare malignancy initiated and sustained by a small minority of T memory stem cells transformed by human T-cell lymphotrophic virus type 1 (HTLV-1) (562). However, the existence of long-lived memory stem cells has been shown in a variety of human situations, including retrovirally modified clones tracked over a decade post bone marrow transplantation without evidence of malignant clonality (2, 563).

Furthermore, a wide variety of experimental strategies have been mooted to enhance the generation of early memory anti-tumour T cells, from metabolic approaches (Akt inhibition) to modification of self-renewal pathways (glycogen

synthase kinase-3β (GSK-3β) inhibition) or epigenetic agents (DNA methyltransferase 3a (DNTM3a)) (187, 192, 518). As yet none of these approaches have yet been translated into clinical success. For example, GSK-3β inhibition also reduces T cell proliferation, preventing generation of sufficient cell numbers (211). Other groups have suggested priming cells ex vivo in the presence of low dose IL-7 and IL-15 can generate greater number of T memory stem cells but numbers remain limiting (564). Rapid good manufacturing practice (GMP)-compliant generation of cells capable of immediate tumour lysis is one of the major rate-limiting steps preventing effective translation of adoptive T cell therapy. Using a pre-existing homeostatic BM niche to dramatically amplify cell numbers in vivo could reduce the duration of ex vivo culture, provide rapid ongoing anti-tumour control and prevent induction of senescence.

The financial implications of cellular therapy cannot be ignored, particularly in the light of astronomical price tags placed upon the first CAR-T cell products to market. Whilst these companies have undoubtedly accelerated commercialization with the use of closed automated systems and large-scale manufacturing, a lack of investment and input into initial development has been a major reason for the wasted decades before the potential of immunotherapy was realized. The majority of people in middle-income and developing countries will be unable to access these therapies using current pricing strategies. The growth of high-cost precision medicines tailored to very small populations has only exacerbated this issue. In the United States the launch price of oncology drugs per life-year gain has quadrupled within two decades, whilst the number of new drugs approved per billion dollars spent

has halved every nine years since 1950 (565). The current unsustainable trajectory presents us with the possibility that effective drugs, particularly cell therapies, will only be developed for an increasingly small market able to afford them. With this in mind, there is a new imperative for alternative models for bringing drugs to market, potentially through public ownership of not-for-profit companies spun out of academia. The Organisation for Economic Cooperation and Development (OECD) and government ministers in the Netherlands have recently called for new solutions to this problem, such as agreements linking price to innovation, research funding input and long term outcomes (566, 567), and it is important that academics and clinicians engage with and drive these discussions based upon their understanding of patient need.

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